TITLE Cytological Phenomena Observed During Selective and Nonselective Injury to Malignant cells in Tissue Culture with Penicillium Extracts ...

AUTHOR Ivor Corryman

UNIVERSITY of Michigan DATE 1949

DEGREE Ph.D. PUBLICATION NO. 1241

CYTOLOGICAL PHENOMENA OBSERVED DURING SELECTIVE AND NONSELECTIVE INJURY TO MALIGNANT CELLS IN TISSUE CULTURE WITH PENICILLIUM EXTRACTS AND WITH NITROGEN MUSTARDS.

Ivor Cornman

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan.

June 1949

Committee in Charge:

Prof. A.F. Shull, Chrm.
Prof. W.C. Steere
Prof. P.O. Okkelberg
Prof. F.H. Test
Prof. A.E. Woodward

CONTENTS

INTRODUCTION

In a cancer research program, tissue culture can make two paramount contributions. First, a research into the etiology and characteristics of the malignant transformation enables one to isolate and study the neoplastic cell, or even to change a cell from normal to neoplastic outside the animal body (Earle 1947, Gey and Gey 1947). Second, in the search for chemicals to be used in the therapy of cancer, the naked mammalian cells can be exposed directly to the compounds tested, thus avoiding the obstructions of somatic toxicity or detoxification which complicate in vivo studies.

The first type of contribution is essentially descriptive. One can list and describe the qualitative changes seen, and follow the sequence of events in the living cells. The second, the screening of substances for possible chemotherapeutic value, requires a quantitative approach. Before one can definitely state that a substance is or is not worth further testing, he must establish criteria of damage. He must find a repetitive pattern by which both normal and malignant cells reveal injury and then establish an objective scale by means of which the degrees of damage can be compared. He thus reduces the millions of decisions about thousands of cells in hundreds of cultures to a few numbers which tell whether one compound is better than another by the standards established in the test.

A resort to tissue culture necessarily removes the results one step from the whole-animal experiments which give more definitive answers as to chemotherapeutic value, but tissue

culture is to be regarded as a sensitive feeler to indicate the direction which the final therapy may follow. Tissues in culture may reasonably be expected to reveal the action of a substance comprising a minute portion of a complex mixture, or reveal a weakly active homologue of the compound which may later exhibit a therapeutic value.

The substances chosen for this attempt to establish tissue culture as a screening technique were crude Penicillium extracts and fractions as examples of heterogeneous mixtures, and the nitrogen mustards as examples of a large family of compounds known to be extremely active biologically.

Of the various culture methods the roller-tube technique (Gey 1933, 1936) offers two main advantages in that up to 20 fragments of tissue can be planted, thus permitting an adequate sampling of the tissue to be tested, and also because normal and malignant tissue can be grown side by side, so that there can be no question as to differences in the composition or concentration of the medium reaching the two types of tissue. This exact control is requisite in tests that are necessarily conducted at threshold concentrations where slight differences in dosage give considerable differences in effect.

The initial work with crude penicillin in these studies was carried out at the Wistar Institute and financed by a grant to Dr. Warren H. Lewis from the International Cancer Research Foundation. To Dr. Lewis the author is deeply indebted, and also to Dr. Margaret Reed Lewis for her gracious advice and assistance.

Recent work on Penicillium filtrates and penicillin fractions, and the nitrogen mustard studies were conducted as a part of the chemotherapy screening program at the Sloan-Kettering Institute.

Because of the extreme diversity of the substances studied, these two groups of substances are considered in separate sections.

PENICILLIUM EXTRACTS

PERTINENT LITERATURE

There would be little purpose to outlining the history of penicillin and its many clinical uses, inasmuch as the work of Dr. M.R. Lewis has shown that the factor affecting sarcomas in tissue culture is separate from the bacteriostatic agent. Several reviews are already available: Herrell 1945, Waksman 1945, Fleming 1946, Merck & Co. Bibliography 1946. Work antedating Fleming has been surveyed by Brunel 1944.

Experimental work with "impurities" from Penicillium culture fluid which has appeared subsequent to the publication (Cornman 1944 a,b) of the initial findings of the selective effect reported here will be discussed later.

INITIAL EXPERIMENTS

Materials and Method

Rats of the King A and of the Wistar albino strains, and mice of the black (C_{57}) and the Bagg Albino (B.A.) strains were used. The A strain was kindly supplied by Dr. Helen Dean King. Each of these inbred strains had proved to be 100 per cent

susceptible to the grafts of sarcomas that had been induced in the strain (M.R. Lewis and Lichtenstein 1937). Six rat sarcomas (King A No. 11, No. 89, No. 104, No. 120, and No. 132, and Wistar No. 304) and two mouse sarcomas (C_{57} No. 350 and B.A. No. 37) were used in the cultures. These spindle cell sarcomas had been induced by subdermal injection of dibenzanthracene or benzpyrene (W.H. Lewis 1939). The normal fibroblasts were derived from fragments of muscle from rats or mice, 1 or 2 days old, of the tumor-host strain.

Roller-tube cultures (Gey 1933, 1936 and Lewis 1935) with usually 8 to 10 fragments of a tumor and an equal number of muscle fragments 1 to 2 mm. in diameter were grown in a medium composed of 2 drops of chicken plasma, 2 drops of chick embryo extract, 5 drops of human placental serum and 7 drops of Locke's saline solution. The pipettes used measured 18 to 20 drops to the cubic centimeter. Extensive outgrowth was obtained in 24 to 72 hours, and at this time a record was made of the extent of growth. The initial medium was then replaced by a medium of 7 drops of Locke's solution, 5 of serum, and 2 of plasma, and, in the experimental tubes, 1 to 3 drops of penicillin solution were substituted for an equal quantity of locke's solution. The penicillin solution was prepared from Squibb or Reichel sodium salt of penicillin dissolved in 0.85 per cent sodium chloride, and filtered through a sterile Seitz filter. Such pharmaceutical penicillin preparations contain substances in addition to the penicillin.

The duration of exposure was varied, dosage usually being continued until a definite selective effect was observed. After the effect of the drug had been studied, the penicillin medium was replaced by a Locke's solution plus serum plus plasma medium, and the recovery processes were studied. In those instances where the injured tumor explants showed renewed growth, half the explants were implanted into an animal of the strain native to the sarcoma to test whether the viable cells were malignant or stromal (host) cells. In all, 38 tubes, treated and untreated, were studied.

Results

The malignant cells were consistently more injured than the normal ones (Figs. 1 and 2, 4 and 5). With adequate dosage it was found possible to damage and kill the out-growing cells of all six rat tumors and one of the mouse tumors without killing the cells which grew from the fragments of normal muscle. These malignant cells first reacted to the penicillin by assuming a granular, opaque appearance, with or without vacuoles. This was the initial response to a heavy dose of the drug or the full extent of response to a threshold dose. If the penicillin was removed at this point, all cells recovered. In the higher concentration, along with increasing granularity and darkening of the cytoplasm, there was a retraction of the elongate processes, producing cells irregularly rounded. Upon prolonged exposure the cells disintegrated. Even if penicillin was removed after the cells had rounded up, some

of the cells never recovered. This sequence of changes is not peculiar to penicillin, but is the usual response to many cytotoxins and moderately toxic compounds. In the higher concentrations of penicillin, the normal fibroblasts followed the same sequence of changes. However, there was the very important difference that a concentration of penicillin sufficient to cause the rounding up of some of the fibroblasts, in most instances caused the death of all the malignant cells. A dose too weak to produce any visible cytological changes was nevertheless selective in that it inhibited growth of the malignant explants, while growth of the normal explants was unaffected. In the untreated control tubes the outgrowth of the sarcoma equalled and usually exceeded the outgrowth of the normal cells.

To obtain a quantitative statement of the results, damage to the cells can be classified as incipient (granularity of 50 per cent or more of the cells, and increased irregularity and refractility of the cell boundary), marked damage (rounding,

Table I

Number of Explants of Sarcoma Cells and of Normal Fibroblasts Showing Different Grades of Damage. Combined Totals of all Experiments.

	None	Incipient	Marked	Lethal	Total
Colonies of Normal Tissue	112	92	57	0	261
Sarcoma	0	29	208	104	341

coagulation, or disintegration of the cells, short of 100 per cent), or lethal (no living cells visible). Table I shows the totals of explants classified according to their damage. A further subdivision of the comparisons better reveals the extent of the selective effect. In those tubes in which the 112 normal colonies were not at all affected, there were 29 of sarcoma which showed an incipient effect, 114 which showed marked damage (Figs. 2 and 5), and 23 which were dead. In tubes showing incipient effects upon 92 normal colonies, 70 tumor growths showed marked damage and 46 were completely killed. In tubes in which the 57 normal colonies showed marked damage, 24 colonies of tumor were markedly damaged, and 35 were killed. The over-all effect was clear cut. Not only was the malignant tissue damaged more than the normal throughout the series, but in numerous instances the malignant cells were killed when there was no visible effect upon the normal.

These figures include results obtained with tube cultures of rat tumors and of mouse C_{57} No. 350. Rat tumor 120 was less affected than the others, but the selective effect of penicillin upon the malignant cells of this tumor was unquestionable. The behavior of the mouse tumor B.A. No. 37, on the contrary, proved to be so like that of normal mouse tissue in its reaction to penicillin that the presence of a selective effect was doubtful. In 3 tubes there were 10 muscle colonies showing incipient effect, 16 showing marked damage, and 4 dead, as against the cultures of No. 37 which showed 18

markedly damaged colonies and 7 dead. A dose heavy enough to kill the malignant cells had also killed some of the normal, and the slight apparent advantage of the normal is of questionable significance.

The time at which damage appeared varied with the different cultures and doses, but typically, by use of a dose at the selective lethal level, an incipient damage of tumor cells could be detected at 12 hours. There was marked damage at 24 hours, and complete killing of the growth zone at 48 hours. By 48 hours, however, there was sometimes a new growth of tumor cells, already pushing out from the explants. If this reviving growth was then given fresh medium free from penicillin, the tumor cells grew vigorously. If the fresh medium contained penicillin, however, this new growth in turn was killed off. Four to six days (i.e. two to three changes of penicillin medium) were usually sufficient to eliminate all tumor cells from explants 2 mm. in diameter. Then when the medium free from penicillin was added no tumor cells grew out, or if the sarcoma explants were implanted into rats, no tumors formed. With explants of 1 mm. diameter, however, 2 days sufficed to kill the malignant cells, as determined microscopically and by implantation.

The stroma included in the explants of the various tumors responded much the same as did the fibroblasts growing from the muscle fragments, but was perhaps slightly more susceptible than the normal and grew more slowly than either muscle

fibroblasts or malignant cells. In some tube cultures it was possible, without killing the normal cells, to kill all cells in the tumor explants (Rat tumors 104, 132, 304, and mouse tumor $C_{57}350$), but there may not have been any stroma included in these explants. More frequently, apparently dead explants (initial migration zone disintegrating and no new cells migrating from the explants during treatment) recovered enough in a medium free from penicillin to send forth at least a few stromal cells. These long, fusiform cells resembled fibroblasts rather than the stout, multipolar malignant cells.

As a final safeguard against wrongly classifying viable malignant explants as killed, those pieces which showed no growth and those which showed a growth of stroma or of some doubtfully malignant cells (Figs. 2 and 5) were implanted in young rodents of the corresponding inbred strain. The failure of these cells to grow into tumors showed that the estimation of lethal effect had been conservative in that some malignant explants graded as merely damaged failed to produce tumors. Colonies in 4 tubes graded as 100 per cent lethal, failed to produce tumors when implanted into animals; and out of 21 tubes graded as probably containing surviving tumor cells, only 6 contained tissue capable of producing tumors. To verify the susceptibility of the control animals, they were given implants of untreated as well as the penicillin-treated tumor tissues.

In 2 cultures the dosage in Oxford units was determined by bacteriological assay of the penicillin solution (Cornman

1944 b). Rat tumor 304 was killed at a level of 59 units per cc. of Reichel lot 10533. The muscle fibroblasts in these cultures showed marked damage. The malignant cells of rat tumor 132 were markedly damaged (without any damage to the normal fibroblasts) by 75 units per cc. of Squibb control 87225 and by 73 units per cc. of Squibb control 91478.

Conclusions

The evidence showed fairly conclusively that the agent producing the selective damage was in the penicillin preparations. The effect increased with the increase in dosage, while in control tubes, identical except for the lack of penicillin, the malignant cells grew at least as well as the fibroblasts.

Consideration must be given to the possibility that the medium favors the growth of the cells derived from the muscle, and that penicillin acts by merely lowering the life-supporting powers of the medium, whereupon the sarcoma succumbs first. The sustained superiority of growth of the malignant cells over the normal, however, indicates that the medium was entirely adequate. Omitting plasma or adding embryo extract during the penicillin treatment did not eliminate the selective lethal effect. Tests with different media may prove fruitful, however, in revealing whether penicillin acts upon an intrinsic peculiarity of malignant cells or merely upon a susceptibility created *in vitro*.

CURRENT RESEARCH: SCREENING

In order better to characterize the selective agent in crude penicillin, numerous Penicillium filtrates and fractions of crude penicillin were subjected to a screening procedure.

At this point one must institute a system of objective evaluation whereby many preparations can be tested and compared to replace the slower exploratory method of repeatedly dosing a few cultures to obtain selective killing.

Method

The roller-tube method as modified by the Geys was used throughout. The tissues were held in place by a chicken plasma clot. The standard supernatant nutrient medium was composed of 2 parts of balanced salt solution, 2 of human placental serum, and one of embryo extract, (half embryo, half balanced saline). The serum component was sometimes modified to decrease the amount of lysis (Goldhaber, Cornman, and Ormsbee 1947).

Two or three rows of 8 to 10 fragments were planted in each tube. Usually one row comprised fetal skin and the others were fragments of one or two tumors. When two tumors were grown in the same tube, they were taken from different strains of mice to avoid contamination upon bioassay.

After 24 hours growth in normal medium, each fragment was graded as to vigor and cytological condition. Fresh medium was added, containing known dilutions of Penicillium filtrate or weighed lyophilized filtrate. Exposure to the

penicillin was usually continued for 24 hours. At the end of this time, damage to the tissue was evaluated and then the fragments were either returned to normal medium for study of the extent of damage and subsequent cytological behavior, or they were inoculated into mice to bioassay the residual malignancy.

The evaluation of effects was made as objective as possible. To do this, growth, lysis of medium, and certain cytological changes were chosen as criteria of effects (Fig. 7). They were divided into 4 successive levels of intensity. Granularity and rounding were graded according to degree, as shown in Fig. 7, and also as to frequency. Here again values of 1 to 4 were assigned when 1-25%, 26-50%, 51-75%, and 76-100% of the cells showed increased granularity or rounding. When these percentages of cells had died and broken down, they were scored as grades of disintegration.

With such a scoring it is possible to estimate just how much alteration has occurred during the 24 hours exposure. Furthermore, since untreated cultures were run simultaneously, one can correct for the changes which occurred in the same tissues exposed merely to the normal nutritive medium. Thus, if we indicate exposed tumor as T_E and untreated tumor as T_C with parallel notations for fetal skin N_E and N_C, with times at 24 and 48 hours, we can formulate the index of damage to skin as $(N_E48 - N_E24) - (N_C48 - N_C24)$. That is, the change in disintegration, granularity, rounding, and inhibition of lysis in the untreated skin during the second 24 hours is

subtracted from the change in the treated skin during the same 24 hours. The difference represents the effect of the penicillin during the 24-hour exposure. The index of damage to the tumor is similarly obtained as $(T_E48 - T_E24) - (T_C48 - T_C24)$. In screening compounds which may have some therapeutic value, we are interested in obtaining greater damage to the tumor tissue. This is gauged by the selective index and is obtained by subtracting the skin index of damage from the tumor index of damage. Where skin is damaged more than the malignant tissue, the selective index is of course negative. Following the 48 hour scoring, each row of malignant tissue was inoculated into a mouse to test the viability of the tumor tissue. The normal tissues were cultured another week and given a final score.

Materials

Fetal mouse skin was used as a source of normal epithelial and mesenchymal cells. The tumors were sarcoma L946AII and carcinoma MA387, both from mice. Not all preparations were tested on both tumors.

For initial tests the filtrate was diluted to $\frac{1}{2}$ and $\frac{1}{4}$ with culture medium. Usually fetal skin was tested alone in the preliminary determination of toxicity (cf. Chart 1). Higher dilutions were tested when the filtrate proved active, or when it was too toxic. Dr. Chester Stock supplied filtered fluid from Penicillium (tentative identification) cultured under a variety of conditions (No. 38). One set of fractions was prepared by Dr. J.F. Mahoney of the

V.D. Laboratory, Marine Hospital, U.S. Public Health Service. These included crude filtrate from Penicillium notatum and various components obtained by precipitation and adsorption. Fractions 261, from P. chrysogenum (?) were supplied by Merck & Co. The methods of extraction have not been revealed.

Results

Analysis of a large number of experiments showed that if all selective indices were grouped regardless of the chemical used, most were included within that part of the curve ten points on either side of the zero. The distribution is skewed to the right, giving a few selective indices beyond forty. Accordingly, a selective index of ten was judged insignificant. In the range eleven to twenty we believe the selective effect is doubtful but worth further testing. Indices from twenty-one to forty we consider as indicating that the substance is significantly selective.

The majority of the Penicillium derivatives tested negative (S.I. 10 or less). All four from the U.S. Public Health Service were inactive, as were two preparations of 229. The latter is of special interest -- and disappointment -- because the strain of Penicillium is that which Meyer (1945) used for damaging tumor tissue in vitro.

Seven fractions of Penicillium 261 were also negative, but one fraction displayed doubtful activity: a selective index of 14 at a 5% concentration.

Penicillium grown at Sloan-Kettering yielded some positive filtrates, depending on the conditions of culture. Number 38 yielded six that were negative, two that were doubtful, and two that were positive.

The fractions giving high selective indices did not kill the malignant tissue completely. When reimplanted in mice, the treated explants formed tumors.

The data for filtrate 38d are given in detail in Charts 1 and 2. First the approximate toxic level was determined by exposing skin to media containing 25% and 50% of the filtrate. The damage score jumped from 10 at the beginning of the exposure (24 hours) to 17 and 21 at the end (48 hours).

A lower concentration was then chosen to test for selective activity in tubes containing both fetal skin and tumor L946AII. Tube 3 had 6 fragments of skin and 6 of tumor L946AII; tube 4 had 4 of skin and 5 of tumor.

After 24 hours, both the tumor and the skin explants scored 9 to 10. Growth is recorded, but not included in the damage score. At the end of 48 hours, after 24 hours exposure to 5% of the filtrate, the score for the skin was still low, but the tumor score had risen to 14 to 18 per explant. Totaling the 24-hour score for tube 3 we get 60, and 66 for 48 hours. Its total damage is then 6. The tumor, however, went from 57 to 99, a damage of 42. The control tube, containing no penicillin, did not change during the second 24 hours, therefore the individual figures have not been listed in a chart. Thus the change induced in the skin by penicillin in tube 3 is

SCREENING CHART NO. 1

Preliminary determination of toxicity, using fetal mouse skin, and Penicillium 38d diluted 1:2 and 1:4.

Date March 18, 1947.

Substance tested 38d

24 hour Frag #	25% Tube # 15 1	2	3	4	5	6	50% Tube #16 1	2	3	4	5	6.
Growth	4	3	2	3	3		2	4	4	3	4	
Inhib.Lys.	4	4	4	4	4		4	4	4	4	4	
% Rnd.	0	0	0	0	0		0	0	0	0	0	
Amt. Rnd.	0	0	0	0	0		0	0	0	0	0	
% Gran.	4	4	4	4	4		4	4	4	4	4	
Amt.Gran.	2	2	2	2	2		2	2	2	2	2	
Disint.	0	0	0	0	0		0	0	0	0	0	
Total:	10	10	10	10	10		10	10	10	10	10	
48 hour Frag #	1	2	3	4	5	6	1	2	3	4	5	6.
Growth	4	3	2	3	3		2	4	4	3	4	
Inhib.Lys.	4	4	4	4	4		4	4	4	4	4	
% Rnd.	3	3	3	3	3		4	4	4	4	4	
Amt. Rnd.	3	3	3	3	3		3	3	3	3	3	
% Gran.	4	4	4	4	4		4	4	4	4	4	
Amt.Gran.	3	3	3	3	3		3	3	3	3	3	
Disint.	0	0	0	0	0		3	3	3	3	3	
Total:	17	17	17	17	17		21	21	21	21	21	

Conc. for Screening

Remarks:

BIOASSAY:

Animal #	Tube #	Date and Result	Date and Result	Date and Result

SCREENING CHART NO. 2

Damage scores for fetal skin and tumor L946 exposed to Penicillium 38d filtrate diluted 1:20.

Dept. 107 Tissue Culture Laboratory

Date March 19, 1947

Substance Tested 38d

Tube # 3 Conc. 5% Normal L946A II

Tube # 4 Conc. 5% Normal L946A II

	Tube 3						Tube 4					
24 hour Frag.	1	2	3	4	5	6	1	2	3	4	5	6
Growth	3	3	3	3	3	3	4	4	4	4	4	3
Inhib.Lys	4	4	4	4	4	4	3	4	3	4	3	4
% Rnd.	0	0	0	0	0	0	0	0	0	0	0	0
Amt. Rnd.	0	0	0	0	0	0	0	0	0	0	0	0
% Gran.	4	4	4	4	4	4	4	4	4	4	4	4
Amt. Gran.	2	2	2	2	2	2	2	2	2	2	2	2
Disint.	0	0	0	0	0	0	0	0	0	0	0	0
Total:	10	10	10	10	10	10/60	9	10	9	10	9	10/57
	2	3	3	3			4	4	4	4	4	
	4	4	4	4			4	4	4	4	4	
	0	0	0	0			0	0	0	0	0	
	0	0	0	0			0	0	0	0	0	
	4	4	4	4			4	4	4	4	4	
	2	2	2	2			2	2	2	2	2	
	0	0	0	0			0	0	0	0	0	
	10	10	10	10			10	10	10	10	60	
	4	4	4	4	4	4						
	4	4	4	4	4	4						
	0	0	0	0	0	0						
	0	0	0	0	0	0						
	4	4	4	4	4	4						
	2	2	2	2	2	2						
	0	0	0	0	0	0						
	10	10	10	10	10	10/60						
48 hour Frag.	1	2	3	4	5	6	1	2	3	4	5	6
Growth	5	3	4	4	4	4	4	5	4	5	5	
Inhib.Lys	4	4	4	4	4	4	4	3	4	4	4	
% Rnd.	0	0	0	0	0	0	4	4	2	4	2	2
Amt. Rnd.	0	0	0	0	0	0	2	2	3	2	3	3
% Gran.	4	4	4	4	4	4	4	4	4	4	4	4
Amt. Gran.	3	3	3	3	3	3	3	3	3	3	3	3
Disint.	0	0	0	0	0	0	0	1	0	0	0	0
Total:	11	11	11	11	11	11/66	17	18	15	17	16	14/99
	4	4	4	4	4	4						
	4	4	4	4	4	4						
	0	0	0	0	0	0						
	0	0	0	0	0	0						
	4	4	4	4	4	4						
	3	3	3	3	3	3						
	0	0	0	0	0	0						
	10	10	10	10	60							
	4	4	4	4	4	4						
	4	4	4	4	4	4						
	0	0	0	0	0	0						
	0	0	0	0	0	0						
	4	4	4	4	4	4						
	3	3	3	3	3	3						
	0	0	0	0	0	0						
	16	16	15	15	14	91						
6 day Frag.	1	2	3	4	5	6	1	2	3	4	5	6
Growth	5	4	5	5	5	4						
Inhib.Lys	4	4	4	4	4	4						
% Rnd.	0	0	4	0	0	4						
Amt. Rnd.	0	0	1	0	0	2						
% Gran.	4	4	4	4	4	4						
Amt. Gran.	3	3	3	3	3	3						
Disint.	0	0	0	0	0	0						
Total:	11	11	16	11	11	17						
	5	4	5	4								
	4	4	4	4								
	4	4	4	4								
	1	1	1	1								
	4	4	4	4								
	3	3	3	3								
	0	0	0	0								
	16	16	16	16								

BIOASSAY.

Animal	Tube #	Date & Result	Date & Result	Date & Result
10	3	4-3-47 +		
16	4	4-3-47 +		

Remarks:

graded at 6 - 0, or 6, while the penicillin-induced damage to the tumor is 42 - 0, or 42. The selective damage to the tumor is then 36. The totals in tube 4 are calculated on a basis of 6 explants, from the averages of the 4 and 5 explants present. Here the skin stayed at 60, while the tumor went from 60 to 91. No adjustment need be made for the control tube, since its change was zero, so the selective index for tube 4 is 31, and the average for the two tubes is 34.

Not all the tumor cells were killed, despite the high selective index, as shown by the positive bioassays. The cells from the skin continued healthy for a week in tube 3, but went downhill in tube 4 (6-day reading).

Conclusions

This screening method, although different from the earlier experiments wherein exposure was continued until the malignant cells were killed, also reveals a factor in some Penicillium preparations which selectively damages tumor tissue. The two tests are supplementary in that the first shows that in a complex mixture such as crude penicillin, a substance can be detected and controlled so that it will selectively kill malignant cells in tissue culture, leaving the nonmalignant cells more or less healthy. The second technique enables one to screen several substances simultaneously and quickly evaluate their potentiality for damaging tumor cells, even though the 24-hour exposure does not kill. More intensive tests are now in order, to determine whether the factors detected by the second technique can be made to kill malignant

cells without damaging neighboring normal cells.

SURVEY OF EFFECTS OF PENICILLIN IMPURITIES

Interest in additional physiologically active agents produced by Penicillium has grown in several fields of research.

Damage to Tumor Cells

Meyer (1945), using an in vitro technique which is not selective, i.e. not controlled by simultaneous exposure of normal tissue, was able to render mammary adenocarcinoma inactive on transplantation back into the host strain. Pieces 3-5 mm. in diameter were exposed to crude Penicillium filtrate or to Tyrode solution. Only those in Tyrode were viable. The impurity responsible was stable at 100° C, was adsorbed in carbon, but was not soluble in ether or chloroform.

Reports have come to the author of many futile attempts in different laboratories to induce regression of tumors in a number of experimental animals. It is hard to evaluate such results inasmuch as the composition of the penicillins was not known, and they were not assayed in tissue culture.

However, Beard (1944) obtained regression of EMGE sarcoma in rats. Tumors disappeared completely in some rats where subcutaneous injections of 333 Oxford U./cc. began the day the tumor was inoculated. Injection of 333 or 666 units into rats bearing large tumors induced a decrease in size but did not destroy the tumor.

In nine of twelve mouse mammary adenocarcinomas and one of two sarcomas, there was a decreased rate of growth

during penicillin treatment. Dobrovolskaĭa-Zavadskaĭa (1946 a,b) believes that the accelerated growth of the other three resulted from pathological dilatation of the blood vessels, as there was little mitotic activity. A distinct zone of cellular damage was found in the treated tumors, and this necrosed, but unaffected cells outside this zone resumed growth when the treatment was stopped.

We might note in passing that filtrates from Aspergillus (Kidd 1947) and Sporosarcina (Cohen, Borsook and Dubnoff 1947) are damaging to tumor cells in vitro.

Drs. Mahoney and Arnold at the U.S. Marine Hospital observed regression of a gastric carcinoma following two intensive courses of penicillin treatment totaling 13,500,000 units. Cancerous lesions remaining after surgical resection of part of the affected stomach disappeared, and there was no indication of pathological condition two years later. A low-grade squamous-cell carcinoma in another patient was saturated with penicillin by electrophoresis (following a light course of intramuscular penicillin). The cancer healed in two months and showed no relapse three years later (personal communication).

Inhibition of Mitosis in Normal Cells

Bucher (1947 b), in agreement with M.R. Lewis (1944) found that pure penicillin was nontoxic in tissue culture. Rabbit fibroblasts exposed ten hours to pure penicillin G showed mitotic activity equal to the controls. Commercial

penicillins, on the contrary, inhibited growth of cultures in the range of 100 - 200 Oxford units (Bucher 1946 a,b, 1947 a). The mitotic coefficient first decreased, then increased as metaphase was prolonged. Chromosome orientation on the spindle was retarded, and sometimes the chromosomes became pycnotic, or were scattered through the cell. These manifestations increased when older solutions were tested, unequivocally demonstrating that impurities were responsible. Penicillin quickly decomposes in solution.

Mitosis in cultured hen macrophages was delayed and diminished by addition of 1/500 penicillin (potency not given) to this medium (Jacoby 1941).

Crude filtrate induced a brief leukopenia, then leucocytosis in rabbits (Reggianini and Brillanti 1947). Cleavage of Arbacia eggs was delayed and finally stopped by commercial penicillin at 333 mg/L (Henry and Henry 1945). Clowes has reported that an impurity is responsible for the cleavage-inhibiting action of penicillins he has tested (cf. Burk et al. 1947).

Arbacia eggs exposed to the same penicillins as tested by Burk (cf. below) revealed the same order of potency for mitotic inhibition as he found for metabolic suppression. The most active was his "Penichromin", perceptibly delaying cleavage at 5 mg/L. Most penicillins fell within the range reported by Henry and Henry, retarding cleavage at 300 mg/L. Penicillium 261E and K were the most effective. Liquid filtrates 34K delayed cleavage when diluted 1:10, and 229M at 1:100 (Cornman, unpublished).

Metabolic and Enzymatic Effects

A potent inhibitor of respiration and glycolysis was found in crude penicillin by Burk et al. (1947 a,b), and DuBuy et al. (1947). Destruction of the included penicillin by penicillinase only partly removed the metabolic suppressor. There was no difference in effects on adenocarcinoma, sarcoma, and normal tissues from adults or embryos.

Inhibition of the action of urease by crude penicillin is reported by Scudi and Jelinek (1944), Vargas and Escubós (1945) and Kun (1948). Smolens, McAleer and McLaren (1947) report detoxification of bacterial toxins by crude penicillin. The effects on the frog's nervous system may be related to these findings (Taormina 1946, Kollros 1947).

Effects on Plants and Microörganisms

Mitosis in wheat seedling roots was suppressed by filtrates from Penicillium cultures (Gerola 1946). Keeping in mind the fact that roots are in general inhibited by concentrations of auxin which stimulate stems, this may have some bearing on de Ropp's discovery of a stimulatory effect on proliferation of sunflower stem in culture by commercial but not by pure penicillin. He suggests it may be due to indole acetic acid.

Welch, Randall and Price (1947) have investigated an enhancement factor present in penicillin which renders it more active against bacteria. A comparable factor was reported also by Hirsch (1944). Partially purified penicillin is also more active against Treponema (Dunham and Rake 1945).

Fischback, Eble and Levine (1947) traced the enhancement factor to o-hydroxyphenylacetic acid.

DISCUSSION

While the search for the selective lethal agent -- or agents -- demonstrated to be present in crude penicillin is progressing, it by no means follows a direct or simple course. Even when a strain of Penicillium is known to produce a factor, not every filtrate gives a positive test. Penicillium 38 produced active substances only under certain conditions. Penicillin production is also closely linked to the method of culture. One can reasonably hope that after further experimentation, moulds can be induced to yield more of the anticancer agent, just as penicillin production can be increased under controlled conditions.

In view of the myriad substances produced by moulds, and the many effects already recorded for crude penicillin, we can not at present be justified in assuming that the selective anticancer agent found in different Penicillium preparations is one compound.

There is already good evidence, reviewed in the preceding section, that indole acetic and hydroxyphenylacetic acids are in part responsible for the suppressive effects of crude penicillin on proliferation in lower organisms.

Sea urchin eggs are being used in an attempt to obtain correlation of the metabolic effects on one hand, with the anti-cancerous effects on the other, using normal cell division as the middle ground. Correlation between metabolic and cleavage

effects has been good. Correlation with the tissue culture studies has not been impressive, but only a few filtrates have been tested on both eggs and cultures.

NITROGEN MUSTARDS

HISTORY

Inhibition of Cell Division by Mustard Gas: Bis(β-chloroethyl) sulfide

Scattered observations on the effects of sulfur mustard presaged its striking effect on cell proliferation which were discovered later to be typical of all mustards. Granulocytopenia was found to precede death following lethal exposure of human beings or experimental animals to mustard gas, bis(β-chloroethyl) sulfide (Krumbhaar 1919, Warthin and Weller 1919, Flury and Wieland 1921, Richters 1932, Muntsch 1934, Maier 1938, and Drews 1939). Significantly, the effect is exerted whether it enters via the skin or the lungs.

These peripheral blood changes were traced to inhibition of proliferation in the marrow by Krumbhaar and Krumbhaar (1919) in the human organism, and by Pappenheimer and Vance (1920) in rabbits. These studies have been extended by Kindred (1946, 1947) who traced the hypoplastic trend in rat marrow. Graef and his coworkers (1946, 1948) have described marrow depletion in mammals and birds.

Lymphocytopenia is also revealed by the more complete studies of peripheral blood changes. Its origin in inhibition of mitosis in the rat thymus and lymph nodes has

been demonstrated by Kindred (1947). Graef and his coworkers (1946, 1948) emphasize the abruptness of the effect since there is lymphocytic fragmentation and loss of weight in the thymus, lymph nodes and spleen as early as ten hours after heavy dosage.

The prominent alterations found in the hematopoietic system do not reflect a selective response other than on the rate of proliferation. Proliferation was also halted in the cornea (Friedenwald, Buschke and Scholz 1948) and the intestinal epithelium (Friedenwald and Scholz, Ref. 54 in Gilman and Philips). Mitosis in regenerating rat liver dropped sharply after sulfur-mustard administration in doses which did not decrease the phosphorus uptake by liver cells (Marshak 1946). Proliferation in the basal layers of mouse skin was indefinitely suppressed by repeated application of sulfur mustard (Fell and Allsopp 1948 b). Chick embryo fibroblasts in culture ceased dividing when the concentration reached 500 mg/L (Fell and Allsopp 1948 a).

Lillie, Clowes and Chambers (1919 a,b) conducted the first experiments on inhibition of division in echinoderm eggs (Arbacia and Asterias). They emphasized the long latent period between the short exposure and the eventual disruption of cleavage.

Mitosis is also inhibited in plants as revealed by the studies of Koller on mitotic and chromosomal abnormalities in Tradescantia pollen grains (Ref. 57, Gilman and Philips), and by the observations of Kinsey and Grant (1947 a) on yeast

cells.

Despite these suggestive leads, and speculation on selective damage to the nucleus comparable to x-ray effects (Flury and Wieland 1921), there appears to have been no early attempt to destroy malignant growth with the sulfur mustards. Recently Bass and Freeman (1946) induced regression of mouse lymphoma and lymphoid leukemia with bis-(β-chloroethyl) sulfide. However, a myelogenous leukemia, mammary carcinoma, spindle cell carcinoma and a malignant melanoma did not respond.

Inhibition of Cell Division by Nitrogen Mustards

With the advent of studies on nitrogen mustards, the leukopenic effect of mustards was rediscovered. Most results of experiments in which it was found that nitrogen mustards displayed the same damaging effect upon myelogenous and lymphatic proliferation as does mustard gas are not yet available in the open literature (cf. Gilman and Philips 1946).

The results of Graef, Karnofsky and Smith with ethyl bis-, methyl bis-, propyl bis- and tris-β-chloroethyl amine, were reported briefly in 1946. In their later paper (1948) the effects of HN2 on the blood and marrow in rats, mice, rabbits and hens are described in detail. There was early leukocytosis with the lymphocyte count dropping first, then the granulocyte. Hematopoietic cells in the sternal and femoral marrow of rats and rabbits began to degenerate within eight hours after injection of lethal doses. Cells and nuclei became enlarged, and some were completely destroyed. Mitoses

had ceased after eight hours. Thereafter, depletion of the marrow progressed rapidly to almost complete aplasia in forty to ninety hours. All cell types decreased simultaneously except the persistent megakaryocytes with bizarre pycnotic nuclei.

Depletion by simultaneous cell destruction and mitotic inhibition was also the typical picture in lymphoid tissue. The lymphocytes fragmented, usually by karyorrhexis, and the germinative centers in the nodes, spleen, and thymus were lost. Lymphoid tissue lost about a fourth of its original weight on each of three successive days after injection of HN2. Degenerative and inflammatory changes in the intestine appeared so early that no specific effects on proliferation were discernible. Meiosis in the testes did not appear to be affected.

These results are almost completely in agreement with the blood studies of Kindred (1947), who found lymphopenia and granulocytopenia with HN2, but only lymphopenia in HN1- and HN3- poisoned rats. Significantly, thymus lymphocytes *in vitro* showed changes like those *in situ*. HN3 at 1 mg/L produced nuclear damage in two hours. The nucleus became opaque, then the chromatin broke into small vesicles which later flowed together, and finally the nuclear membrane seemed to dissolve.

Schrek (1947) has also reported on *in vitro* changes in myeloid and lymphatic cells treated with nitrogen mustard.

Cells *in situ* which permit more nearly direct application of the mustard are those of the cornea. Friedenwald, Buschke and Scholz (1943) showed that inhibition of epithelial mitosis began at 70 minutes. Mitoses reached a minimum at 18 hours, then increased, overshooting the control counts at 40 to 50 hours, when 3.7 mg/L of HN2 was dropped into the eye of a rat. The cells continued to increase in size throughout the experimental period.

This growth of cells deprived of proliferative power was dramatically demonstrated by Gillette and Bodenstein (1946) and Bodenstein (1947 a,b) in embryos of *Ambystoma maculatum*. Exposure to 10 mg/L of HN2 for 45 minutes stopped all mitosis, but permitted the cells of the eye, nervous system, intestine, and skin to differentiate and grow to many times the normal size.

Exposure of sea-urchin eggs to HN2 before or after fertilization slowed or blocked cleavage (Cannan and coworkers quoted by Gilman and Philips, Ref. 52, and by Barron *et al.* 1948). Barron's group reported that exposure of the sperm to 10^{-3} M HN2 before fertilization also slowed cleavage, and 10^{-4} M dosage resulted in delayed blastulation and abnormal plutei.

Division of *Chilomonas*, a unicellular plant, was inhibited by 1.9×10^{-5} M HN3 (Hutchens and Podolsky 1948). Yeast multiplication was inhibited by 5×10^{-8} molar HN2 (Kinsey and Grant 1947 b).

Nitrogen Mustards in Chemotherapy of Cancer

That the leukopenic action of nitrogen mustard was so quickly seized upon, in contrast to the way in which this therapeutic lead was ignored when it was first discovered with sulfur mustards, can almost certainly be ascribed to the current optimism in the chemotherapeutic attack on cancer. The pioneer work by Gilman, Goodman, Lindskog, and Dougherty in 1942 (Gilman and Philips, Ref. 65) was followed by intensive clinical studies in many laboratories. The response of the normal blood picture to the mustards was like that in experimental animals.

Typically, following injection of the methyl-bis- (Jacobson et al. 1946, Spurr et al. 1947) and tris-β-chloroethyl amine (Rhoads 1946) granulocytopenia followed lymphocytopenia. In the marrow, the myeloid series were damaged first, and there followed a depression of the erythroid series. The impression was gained of a special sensitivity of premyelocytic stages, the myelocyte maturing but not dividing under the influence of the mustard (Spurr 1947).

The leukopenic action of methyl-bis and tris was found to operate in reducing the cell count and clinical symptoms of chronic lymphatic, and to a lesser extent, of myelogenous leukemias (Jacobson et al. 1946, Goodman et al. 1946, Rhoads 1946, Spurr et al. 1947, Karnofsky et al. 1947). Wilkinson and Fletcher (1947) were more successful in treating the chronic myelogenous type, however.

Remissions were also reported by the above authors in cases of Hodgkins disease and lymphosarcoma, and in cases of Hodgkins by AnThomas (1947), Taffel (1947) and Hofmeyr (1947).

A newer mustard, 1,3-bis-[bis-(β-chloroethyl)-amino]-propane has been tested at the Memorial Hospital (Erslev et al. 1947). Unlike the simpler mustards, this tetrakis structure consistently reduced the cell count in acute, as well as in chronic myelogenous leukemia, but not in chronic lymphatic leukemia.

The effect on tumors not derived from the hematic tissues has not been consistent as with leukemias (Hawkins and Farber 1947; Boyland, Koller and Warwick 1947).

Therapy of leukemias and tumors in mice has yielded quantitative data which can best be considered later in the discussion.

Although malignant proliferation is thus extensively stopped, there appears to be no selective action of the nitrogen mustards upon cancer cells (Rhoads 1946). Rather, cells are affected in proportion to their rate of proliferation (Gilman and Philips 1946).

Alteration of Mitosis by Mustards

There appears to be enough similarity in the cytological effects of sulfur mustard and nitrogen mustards to justify their consideration together.

Retardation of mitosis is inferred by Friedenwald, Buschke and Scholz from the presence of 134-110% mitoses in corneal epithelium exposed to 0.5 - 0.03 mg/L HN_2, as compared with counts in the control eyes. Another indirect measure of retardation of division is available from the work of Kinsey

and Grant (1947 a,b). The average rate of proliferation of yeast cells was decreased for several generations after a few hours exposure to M/1000 and M/500 sulfur mustard. However, the visible heterogeneity of the population after treatment leaves open the possibility that some cells were proliferating normally while others were not budding at all.

Direct observation of slowing of mitosis in one population of cells is possible with marine eggs. Timing of fifty per cent cleavage of Arbacia eggs revealed that retardation is a typical effect of many nitrogen mustards. Continuous exposure to HN2 at 0.5 mMolar, HN3 at 0.04 mMolar and bis- [bis-(β-chloroethyl)-amino]-ethane at 0.3 mMolar increased the time between first and second cleavages 200 - 400%. Interphase and formation of the mitotic figure were both prolonged (Cornman, unpublished).

Earlier studies with marine eggs, using short exposures of the fertilized or unfertilized egg (cf. Gilman and Philips 1946 and Philips and Gilman 1947) revealed a specific interphase block which was not readily apparent in the above experiments, wherein the eggs were continuously exposed.

Morphological changes seen in mouse epidermis repeatedly exposed to sulfur mustard included clumped and lagging chromosomes, polyploidy, multipolar spindles and multinucleation (Fell and Allsopp 1948 b). In rat corneal epithelium, there was pycnotic fragmentation of the nucleus in proportion to the mitotic activity. Friedenwald and Buschke (1948) suggest that a premitotic state is selectively affected by the mustard, while later stages continue to completion.

Meier and Schär (1947), on the other hand, exposing chick fibroblasts to 0.01 mg/L HN2 in tissue culture, found that the prophases were normal, but thereafter the chromosomes were scattered without orientation on the persistent spindle. Fell and Allsopp (1948 a) exposed chick fibroblasts to sulfur mustard, and found abnormal mitoses most conspicuous at 50 and 100 mg/L. These included spindles that were multipolar or eccentrically situated, chromosomes that lagged or fragmented, and multinucleation.

Sulfur mustard in regenerating rat livers prevented cells from initiating mitosis, while cells in metaphase and anaphase apparently continued to completion. No chromosomal aberrations were found (Marshak 1946).

Koller (1947) found the cytological effects of nitrogen mustards and ionizing radiations closely comparable. Both induced stickiness and lagging of chromosomes, incomplete spindle formation, and suppression of mitosis in the Walker rat carcinoma. Koller, Ansari, and Robson (Ref. 57, Gilman and Philips) also induced chromosome breaks in Tradescantia pollen mitosis with sulfur mustard, a radiomimetic phenomenon of considerable significance. Identical abnormalities were found in the cells of a gastric carcinoma of a patient treated with HN2: chromosomes became fragmented, clumped, pycnotic, or adhered as anaphase bridges (Boyland 1947).

More subtle chromosomal changes are detectable only genetically. Mustard gas induced mutations in Drosophila (Auerbach 1947, Auerbach and Robson 1947, Slizynska 1947) and

in Neurospora (Horowitz et al. 1946).

This historical summary is not complete in covering mustard research, nor in giving credit to many workers in the field. Complete accounts are not available because of security restrictions, but some references to wartime research are available as personal communications in the more recent articles listed here. Clinical studies which lend weight to the findings of the pioneer therapeutic work, but do not extend the cytological information, as well as the many papers covering the chemical and biochemical behavior of the mustards are outside the scope of this paper and have been omitted.

MATERIAL AND METHODS

Tissues

Most tissues were obtained from mice. The normal tissue was fetal mouse skin, which provided both mesenchymal and epidermal tissue for comparison with sarcomas and carcinomas.

Three sarcomas were used. Sarcoma 180, a tumor which can be maintained in any strain of mice, was grown and bioassayed in CFW albinos. This was the only heterologous tumor used. L946AII, a fibrosarcoma originating in Jackson Memorial Laboratory mice was maintained in C57 blacks, line 6, from Bar Harbor. Earle's in-vitro-induced sarcoma strain L was obtained through the generosity of Dr. Kenneth Algire. This tumor, identified here as 202L, was maintained in C3H mice of a line started with breeders from the National Cancer Institute, kindly supplied by Dr. Walter Heston.

Three carcinomas were also studied. Adenocarcinoma E060 was grown and bioassayed in C57 black, line 6 mice, the strain of origin. Another, classified as an anaplastic carcinoma of the skin (1025) was obtained from Dr. Jabob Furth, along with a lung carcinoma which during repeated transfers in mice has become sarcomalike (MA 387). These were maintained in AK mice, the strain of origin.

A few experiments with HN3 made use of avian tissue for comparison with the responses of mammalian tissues, and strains of mouse and human fibroblasts which had been isolated earlier and maintained in tissue culture.

Nitrogen Mustards

The hydrochlorides of five congeners were chosen, the simplest being methyl-bis-(β-chloroethyl)-amine hydrochloride. Next was tris-(βchloroethyl)-amine hydrochloride. These compounds, usually referred to as HN2 and HN3 in the literature, are represented in formulae I and II, Fig. 8. The others contain four chloroethyl radicals (the tetrakis series). III is bis-[bis-(β-chloroethyl)amino] -ethane dihydrochloride, of which only an impure sample was available; IV is 1,3-bis-[bis-(β-chloroethyl)amino] -propane dihydrochloride; V is 2-chloro-1,3-bis-[bis-(β-chloroethyl)amino] -propane dihydrochloride. These compounds will be referred to by number for the sake of brevity. The compounds were dissolved in neutral physiological sodium chloride and neutralized with $NaHCO_3$ or phosphate-buffered saline 1 to 3 minutes before they came in

contact with the tissues.

Culture Method and Scoring of Effects

The preliminary screening followed the program described for the recent studies of crude penicillins (page 14). Where more data about the cytological changes or about the lethal dose level *in vitro* were needed, the rigid scoring system was not followed, and observations were suited to the nature of the information desired.

RESULTS

Cytological Effects

Effects During 24-hour Exposure

The initial responses of normal and malignant tissue at threshold doses (the effective levels of different mustards are listed in Table II) resembled those seen during the action of most deleterious agents. The cells became more granular and more opaque (the latter resulting probably from the increased granularity). Fibroblasts and spindle cells (including those of the lung carcinoma MA387) rounded up, changing from their fusiform shape (filiform processes) to oval or spherical (lobate processes, or none at all). This rounding has been observed to progress through a shrivelling stage where the cell membrane suggested a slight crenation. Possibly a brief superficial wrinkling always occurred, but had passed into the more smoothly convex contour by the time the cells were observed. The cell became more refractile, possibly because of the increased thickness. Epithelial cells did not round up at any dosage.

Table II Threshold Doses and Type Responses of Normal and Malignant Cells to Nitrogen Mustards.

	Skin	MA387	L946	202L	130	1025	E060
I	$H_3CN(CH_2CH_2Cl)_2$						
Dose Range in mg/L	0.8-64	0.4-50	2-50	4-32	0.1-9.6	4.32	4.32
Lowest Dose to Damage: 24 hrs.	8-16	8-16	12-16	16	0.6-0.8	4-16	8-16
Lowest Dose to Kill: 24 hrs.	16-32	16	16	32	0.8	12-32	16-32
Lowest Dose: Neg. bioassay	16-32	12	16	--	0.4-0.6	--	--
Conc. Yielding Giant Cells	8	32	--	--	--	4	8
Conc. Causing Blisters	--	--	--	--	--	--	--
Number of Explants Tested	304	136	47	78	418	112	191
II	$N(CH_2CH_2Cl)_3$						
Dose Range in mg/L.	2.3-320	1-320	2.8-160	10-100	1-120	2.3-50	10-100
Lowest Dose to Damage: 24 hrs.	10-20	20	10-20	10	2	20?	10
Lowest Dose to Kill: 24 hrs.	30-40	40	20-40	40	5-10	40?	40
Lowest Dose: Neg. bioassay	40	10-20	20-30	40	2-4	15?	40
Conc. Yielding Giant Cells	10	30	--	--	--	20?	10
Conc. Causing Blisters	208	40?	--	--	--	--	--
Number of Explants Tested	952	421	658	145	835	208	144

	Chick embryo: Heart	Brain	Mouse Fibroblasts	Human Fibroblasts
	II $N(CH_2CH_2Cl)_3$			
Dose Range in mg/L	10-60	10-120	10-40	10-40
Lowest Dose to Damage: 24 hrs.	10	10	20	40
Lowest Dose to Kill: 24 hrs.	40-30	80	>40	>40
Lowest Dose: Neg. Bioassay	80	80	40 or>	40
Conc. Yielding Giant Cells	40	--	10	10
Conc. Causing Blisters	--	80	--	--
Number of Explants Tested	162	97	26	15

	Skin	MA387	L946	180
IV	$(ClCH_2CH_2)_2NCH_2CH_2CH_2N(CH_2CH_2Cl)_2$			
Dose Range in mg/L	.83-49.5	.83-49.5	1.63-49.5	1.25-15
Lowest Dose to Damage: 24 hrs.	13.2	16.5	13.2-24.8	>15
Lowest Dose to Kill: 24 hrs.	>49.5	>49.5	>49.5	>15
Lowest Dose: Neg. Bioassay	>49.5	16.5	16.5	2.5-5
Conc. Yielding Giant Cells	33	--	--	--
Conc. Causing Blisters	--	--	--	--
Number of Explants Tested	154	71	113	76

	Skin	MA387	L946	180
V	$(ClCH_2CH_2)_2NCH_2CHClCH_2N(CH_2CH_2Cl)_2$			
Dose Range in mg/L	1.8-71.7	7.2-14.4	7.2-71.9	1-8
Lowest Dose to Damage: 24 hrs.	29-53.8	>14.4	17.9	1
Lowest Dose to Kill: 24 hrs.	>71.7	14.4	>71.9	8
Lowest Dose: Neg. Bioassay	>35.8	>14.4	>35.8	4
Conc. Yielding Giant Cells	8.9	--	--	--
Conc. Causing Blisters	--	--	--	--
Number of Explants Tested	121	18	73	150

Concentrations near the lethal level produced a blistering of normal cells of epithelial origin, whether they were from mouse epidermis or chick brain. The blister lifted the membrane in a crescent-shaped protuberance which was non-refractile, presumably being filled with aqueous fluid derived from the cell or from the medium.

Of the malignant tissues, only the lung carcinoma MA387 showed blisters, and in only one experiment. In this manner it behaved like epithelium although the cell shape and growth pattern were those of a sarcoma. At lethal levels (Third Ranks, Table II) granularity and rounding were more severe, and the cells began to disintegrate. Concentrations strong enough to kill instantaneously, fixed the cell without

altering the shape or other cytoplasmic features.

Effects Following Return to Normal Medium

Moderately affected cells recovered normal shape, granularity and refractility. Blisters disappeared if the cell survived. Cell division occurred in such cultures, but we can not say whether the cells showing earlier cytoplasmic changes were those which divided.

Cells mortally affected but not killed during exposure to the mustard continued on the way toward disintegration. The situation was confused at the upper dosage levels by fixed cells which, though dead, retained a normal structure. Continued cultivation was necessary to establish their non-viability. The dose preventing in-vitro survival of normal tissue after removal of the mustard is listed in Table II under Bioassay. Tumor tissue was tested in a similar fashion, as well as by bioassay in mice.

Over the entire range of effective concentrations large cells made their appearance, sometimes during the 24-hour period of exposure (Fifth Ranks, Table II). Mouse dermal fibroblasts grew to giant proportions while maintaining the normal nuclear and cytoplasmic features shown in Fig. 9. This cell can be compared in size with Fig. 10, a photograph of the largest cells found in the control cultures. The cell in Fig. 9 is exceptionally large, measuring about 5.6 mm, but as this photograph and Fig. 11 show, the entire population exhibits a trend toward increased size compared to an average population of normal cells, Fig. 12. In Fig. 13, cells

were traced at a magnification of 200 on centimeter coordinate paper to permit comparison of areas. The largest of the untreated cells (A) averaged 600 sq.μ; cells treated at a medium dose (B) averaged 1700 sq.μ and in the culture given a dose lethal for most cells the average was 2800 sq.μ. Epithelial cells of the skin did not show significant enlargement.

Both carcinomas and sarcomas have yielded cells of abnormally large size following nitrogen-mustard treatment. These large cells did not appear with the frequency and regularity that typified the response of dermal fibroblasts, but it was possible to find them often in treated cultures of carcinoma MA387, and sarcoma 202L. Individual cells of 202L occasionally reached giant proportions (Fig. 14) as compared with the untreated, where even binucleate cells did not get as large (Fig. 15). Carcinoma E060 also yielded cells (Fig. 16) far beyond the size of any found in the controls (Fig. 17).

Chick fibroblasts responded with less frequency and at higher concentrations, but the greatest increase in size found so far was observed in chick fibroblasts (Fig. 18 vs. controls, Fig. 19).

These cytological changes were induced in at least one of the tissues by mustards I, II, IV and V, but at different concentrations for each mustard, as listed in Table II. Mustard III was not thoroughly tested because of its impurity.

Mitotic activity decreased as gigantism increased in any experimental series of graded doses. In the cultures from which photographs 3 and 12 were taken, no cells were

found in mitosis. An occasional abnormal mitosis was found following an intermediate dosage (Fig. 20), but similar tripolar mitoses are also found, though rarely, in untreated cultures.

Relative Activity of Different Mustards

Compared for milligram concentration per kilogram of culture fluid, the toxicity and cytological effectiveness decreased with increase in molecular weight. Mustard III 1,3-bis-[bis-(β-chloroethyl)amino]-ethane, was an exception, barely altering the cell at 256 mg/K, and not lethal even at 512 mg/K, but the sample available was impure.

Converted to millimolarity, the concentrations required to produce a threshold cytological effect were I: 0.051 mM; II: 0.049 mM; IV: 0.033 mM; V: 0.067 mM.

At this point it is advisable to call attention to the instability of the nitrogen mustards in alkaline solution (Golumbic, Fruton and Bergmann 1946; Gilman and Philips 1946). So fast do they react with the medium, that there is always the question of what concentration actually reached the cells, although the experimental medium was washed over the cells within one to three minutes after the mustard was neutralized. It proved impractical to attempt to define effective levels closer than by a factor of two.

Susceptibilities of Different Tissues

With one exception, all tissues, normal and malignant, were equally susceptible to the nitrogen mustards insofar as

this method of culturing and evaluating permits one to determine. The exception was sarcoma 180, which is peculiar among the test tumors in that it is heterologous. Both in-vitro visible damage and in vivo behavior subsequently (bioassay) showed a sensitivity at one tenth the concentration which affected other tissues. Table II shows that the higher susceptibility obtained, whatever the mustard used, except for in-vitro damage with compound IV.

Mouse and human fibroblasts maintained in cultures several months were as sensitive as mouse fibroblasts growing from freshly explanted fetal skin. Proliferating chick heart or brain, on the other hand, survived doses twice that which killed normal mouse cells, and 40 times that rendering sarcoma 180 ineffective.

The mustards, then, distinguish between cells of widely different animals, or they can enhance the latent incompatibility of host and heterologous tumor (S 180) but they give no evidence of a selective action on malignant cells as against host cells.

DISCUSSION

Cytological Effects

Granularity, rounding, and disintegration are non-specific responses of little interest in analysis of the specific effects of nitrogen mustards.

Blistering of epithelial cells is unusual. It was seen earlier in chick fibroblasts by Fell and Allsopp (1948 a) with HN2, but wartime restrictions prohibited publication

(cf. Karnofsky et al. 1947 a). It is perhaps too obvious a comparison to point out that the vesicant action of the nitrogen mustards appears at least superficially similar at the cell surface and at the body surface. However, loss of fluid from the cells may be an essential part of the toxicity pattern, inasmuch as the pharmacological symptoms of mustard poisoning include fluid loss. Observation of the living cells has not revealed whether water was lost through these blisters. They disappeared when the cell recovered, but were not seen to burst.

The growth of cells to extraordinary size has been reported as a typical effect of nitrogen mustards in rat corneal epithelium (Friedenwald, Buschke and Scholz 1948), in amphibian embryonic cells (Gillette and Bodenstein 1946) and in mammalian sarcoma cells grown on the chorioallantois (Karnofsky et al. 1947 a). All these studies point to a fairly general propensity of the nitrogen mustards for causing proliferating cells to enlarge instead of dividing: mouse normal, sarcomatous and carcinomatous tissues, and chick normal tissue. The enlarged cells are typically uninucleate, and while unable to divide, are able to differentiate -- in amphibian embryos at least. Mouse fibroblasts maintain their normal shape despite enormous increase in size. They are not "giant cells" in the sense used by the pathologist, encompassing reaction cells which are usually round and multinucleate.

In tissue culture, large cells begin to appear at concentrations which decrease the mitotic count. At higher levels, where most of the cells are larger than normal, no mitoses remain. No giant cell has been found which shows any evidence of mitosis. To all appearances, the sequences of synthesis and growth persist in the absence of ability to proliferate. The response within any population of cultured cells is erratic. Some cells continue to divide while others grow. At higher doses, some cells are killed, while others, presumably those protected within the explant, persist and enlarge. The large cells do not survive more than two weeks in the experiments with prolonged culturing in normal medium, nor can they be transplanted as roller-tube or slide cultures.

The relationship between cell overgrowth and inhibition of division has been discussed recently by Eisenstark and Clark (1947) as it applies to radiation. We are thus able to extend the already numerous comparisons with x-ray effects. The clinical response of leukemias, the mutagenic effects, the breakage of chromosomes, and the long latent period, all are radiomimetic effects reported in the literature discussed in the preceding pages.

Comparative Effectiveness of the Mustards

On a molar basis, the mustards are about equivalent in producing threshold cytological damage. This is not the case when the LD_{50} for mice is converted to millimols

(unpublished data supplied by Dr. Joseph Burchenal and by the Army Chemical Department, Edgewood Arsenal):

		Cytological mM	LD_{50}
I	Methyl-bis(β-chloroethyl)amine HCl	.051	.027
II	Tris(β-chloroethyl)amine HCl	.049	.009
IV	1,3-bis-[bis-(β-chloroethyl)amino]-propane diHCl	.033	.009
V	2-chloro-1,3-bis-[bis-(β-chloroethyl)amino]-propane diHCl	.067	.039

Anslow et al. (1947) report a comparable higher toxicity of HN3 in comparison with HN2 in the mouse and rat (HN2 being slightly more toxic to the rabbit).

In ability to kill an intact mouse then, only II and IV are equal, I and V being one-third and one-fourth as effective. This underlines the value of the tissue culture method for getting closer to the reaction between cell and experimental agent, by-passing detoxification and "weakest-link" mortality resulting from damage to one system or organ.

It is of theoretical interest to note that neither in-vitro nor in-vivo toxicities substantiate the reasonable expectancy that the tetrakis compounds having two bis-chloroethyl-amino groups should display an effectiveness double that of the bis compounds. Quite probably a molecule can react only once and rarely gets into a position where both ends can combine with a cellular component.

Tissue Susceptibilities

The uniformity with which normal and malignant cells

respond to nitrogen mustards is disappointing, but in keeping with the extensive findings in clinical use. Nitrogen mustards damage proliferating cells, healthy and neoplastic, to the same degree (Rhoads 1946).

Sarcoma 180 is the exception, responding at 1/10 the concentrations required to affect other tumors. The quicker cytological response *in vitro* perhaps only reflects the instability of S 180 under the conditions of culture. It is one of the most difficult tumors to maintain in tissue culture. Negative bioassays following relatively low doses can be compared to the ease with which x-ray confers immunity to this heterologous tumor, but not to homologous tumors (Goldfeder 1945).

The resistance of chick tissue to nitrogen mustards is in keeping with the findings of Karnofsky (Karnofsky *et al.* 1947 a). Sarcoma 180 was killed at 0.1 mg/egg while the 12-day chick required 0.9 mg/egg.

Critique of Tissue Culture as a Cancer Chemotherapy Screening Method

From the point of view of the experimental cytologist the cytological results and toxicity data could reasonably be an adequate *raison d'etre* for the experiments reported here. However, in undertaking such studies, we have posed the question of utility in a chemotherapy program.

Two requirements need to be satisfied to justify the use of tissue culture for the screening of compounds. First, one must demonstrate a selective effect: damage to

malignant tissue in excess of that sustained by normal tissue. Second, one must evaluate the cytological changes so that objective criteria can be applied in the processing of a large number of substances.

These requirements seem fairly adequately met by the penicillin experiments. A selective effect was found, and a scoring system was devised which permitted separation of a few Penicillium filtrates from a larger number of inactive preparations.

The possibility is very great that the selective factor, if it can be isolated, will not be of therapeutic value. One expects many valueless "finds" by each of the numerous screening techniques used in different laboratories. Nor can tissue culture be regarded as the only initial "coarse mesh" in such a screening program. It will not uncover every substance of value. Mustards, for instance, exert no selective effect in cultures, but they have proved to be of very real therapeutic value.

SUMMARY

1. Exploratory experiments exposing normal and malignant tissue simultaneously to crude penicillin reveal a factor selectively lethal to mouse and rat sarcomas.

2. It has been possible, by establishing objective criteria of cellular damage, to show that some Penicillium filtrates and filtrate fractions are more damaging to mouse sarcoma and carcinoma than to embryonic mouse cells.

3. The visible cytological changes are: increased granularity, opacity, rounding and refractility.

4. Nitrogen mustards inhibit proliferation without inhibiting the growth of malignant and embryonic mouse cells.

5. Damaged cells are rounded up, granular, and refractile. Carcinoma and fetal epithelium sometimes show blisters at the cell surface.

6. Effectiveness in mg/L of the mustards decreases with increase in molecular weight. They all produce cytoplasmic alterations and mitotic inhibition at about 0.05 mM.

7. Mouse sarcoma 180 is the most sensitive of the tissues tested. Next come the other sarcomas, the carcinomas, and the embryonic skin, all about equally sensitive. Embryonic chick cells were more resistant than any mammalian cells investigated.

8. The visible cytological effects of crude penicillin and of nitrogen mustards are reversible, but cells which enlarge instead of dividing have not been observed to divide at any later time.

9. Tissue culture can be used to detect substances which selectively damage malignant cells. Criteria of damage can be tabulated to permit an evaluation of a large number of compounds screened by simultaneous exposure to normal and malignant cells.

REFERENCES

PENICILLIN

Beard, H.H., 1944. The effect of penicillin and choline upon the appearance, growth and disappearance of the Emge sarcoma in rats. Exper. Med. & Surg. 2:286-289.

Brunel, J., 1944. Qui a découvert la pénicilline? Rev. canad. de biol. 3:333-343.

Bucher, O., 1946 a. Die Wirkung von Penicillin auf Gewebekulturen. I. Die Beeinflussung der Zellteilung durch Penicillin "Roche". Schweiz. med. Wchnschr. 76:290-293.

Bucher, O., 1946 b. Die Wirkung von Penicillin auf Gewebekulturen. 2. Weitere Mitteilungen über die Wirkung von Penicillin "Roche"; vergleichende Untersuchungen mit Penicillin "Ciba". Schweiz. med. Wchnschr. 76:375-378.

Bucher, O., 1947 a. Die Wirkung von Penicillin auf Gewebekulturen; Untersuchungen mit Penicillin "Lilly". 3. Vergleich der Cytotoxizität verschiedener Penicillin Präparate. Schweiz. med. Wchnschr. 77:171-173.

Bucher, O., 1947 b. Die Wirkung von Penicillin auf Gewebekulturen. 4. Cytopharmakologische Untersuchungen mit reinem Penicillin G. Schweiz. med. Wchnschr. 77:849-852.

Burk, D.; Du Buy, H.; Fischer, C.; Hearon, J. and others, 1947 a. Metabolic inhibitions of microorganisms and animal tissues and tumors by penicillin, penicillin impurity, streptomycin, and cobalt, and some applications to metabolic identification, assay, and mode of action. Progress report at the meeting of the Antibiotics Study Section, Bethesda, Maryland, May 2, 1947.

Burk, D.; Hesselbach, M.L. and Fischer, C.E., 1947 b. The inhibiting action of amorphous and crystalline penicillin and streptomycin preparations on the metabolism of tumors and other tissues. Cancer Research 7:712.

Chambers, R.; Cameron, G. and Kopac, M.J., 1943. Neoplasm studies. XI. The effects in tissue cultures of N,N,N',N'-tetramethyl-o-phenylenediamine and other compounds on malignant lymph nodes. Cancer Research 3:293-295.

Cohen, A.L.; Borsook, H. and Dubnoff, J.W., 1947. The effect of a Sporosarcina ureae preparation on tumor cells in vitro. Proc. Soc. Exper. Biol. & Med. 66:440-444.

Cornman, I., 1944 a. Survival of normal cells in penicillin solutions lethal to malignant cells. Science 99:247.

Cornman, I., 1944 b. A selective lethal effect of penicillin on sarcoma cells growing with normal tissue in roller tube cultures. J. Gen. Physiol. 28:113-118.

Dobrovolskaïa-Zavadskaïa, N., 1946 a. La méthode des controles individuels dans les recherches biologiques de tumeurs, et sa vérification par l'étude de l'effet de la pénicilline. Compt. rend. Soc. de biol. 140:425-427.

Dobrovolskaïa-Zavadskaïa, N., 1946 b. L'effet de la pénicilline sur les tumeurs chez la souris. Bull. Assoc. franç. p. l'étude du cancer 33:192-222.

Du Buy, H.; Burk, D.; Fischer, C.; Hearon, J. and others, 1947. Metabolic inhibitions of tumors and other cells by various antibiotics and cobalt. 4th Internat. Cancer Research Congr. Abstracts p.40.

Dunham, W.B. and Rake, G., 1945. The relative activity of partially purified penicillin and of crystalline penicillin G on Treponema pallidum. Am. J. Syph., Gonor., & Ven. Dis. 29:214-228.

Earle, W.R., 1947. Procedure for the fixation, staining, and mounting of whole mounts from tissue cultures grown in Carrel D 3.5 flasks. J. Nat. Cancer Inst. 8:83-89.

Fischbach, H.; Eble, T.E. and Levine, J., 1947. Ortho-hydroxyphenylacetic acid from an amorphous penicillin. Science 106:373-374.

Fleming, A., 1946. Penicillin; its practical application. Blakiston Co. Philadelphia.

Gerola, F.M., 1946. Escreti fungini e mitosi cellulari. Atti Accad. Naz. dei Lincei, Rendiconti. ser. 8, 1:801-805.

Gey, G.O., 1933. An improved technique for massive tissue cultures. Am. J. Cancer 17:752-756.

Gey, G.O. and Gey., M.K., 1936. The maintenance of human normal cells and tumor cells in continuous culture. I. Preliminary report: Cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation. Am. J. Cancer 27:45-76.

Gey, G.O. and Gey, M.K., 1947. Further observations on the conversion of normal into malignant cells in vitro. Cancer Research 7:729.

Goldhaber, P.; Cornman, I. and Ormsbee, R.A., 1947. Experimental alteration of the ability of tumor cells to lyse plasma clots in vitro. Proc. Soc. Exper. Biol. & Med. 66:590-595.

Henry, R.J. and Henry, M.D., 1945. The effect of penicillin on eggs of the sea urchin, Arbacia punctulata. J. Gen. Physiol. 28:405-413.

Herrell, W.E., 1945. Penicillin and other antibiotic agents. W.B. Saunders Co. Philadelphia.

Hirsch, J., 1943/44. Penicillin Studien in vitro. Compt. rend. ann. et arch. Soc. turque sci. phys. et nat. (12) 1-88.

Jacoby, F. cited in Abraham, E.P.; Chain, E.; Fletcher, C.M.; Gardner, A.D. and others, 1941. Further observations on penicillin. Lancet 241:177-188.

Kidd, J.G., 1947. Effects of an antibiotic from Aspergillus fumigatus Fresenius on tumor cells in vitro, and its possible identity with gliotoxin. Science 105:511-513.

Kollros, J.J., 1947. The convulsive effect of penicillin on an amphibian nervous system. J. Cell. & Comp. Physiol. 29:185-188.

Kun, E., 1948. Inhibition of glycolysis in vitro by impure penicillin. Science 108:117-118.

Lewis, M.R. and Lichtenstein, E.G., 1937. Studies on the transplantability of induced and spontaneous tumors occurring in mice of pure inbred strains. Growth 1:375-383.

Lewis, M.R., 1944. The failure of purified penicillin to retard the growth of grafts of sarcoma in mice. Science 100:314-315.

Lewis, W.H., 1935. Rat malignant cells in roller tube cultures and some results. Carnegie Institute of Washington, Contributions to Embryology 25:161-172, (Pub. No.150).

Lewis, W.H., 1939. Dibenzanthracene mouse sarcomas; histology. Am. J. Cancer 37:521-530.

Merck & Co., Inc., 1946. Brochure with bibliography: penicillin; supplement. Rahway, N.J.

Meyer, J.R., 1945. Indícios da existência de uma substância antineoplásica formada nos líquidos de cultura do "Penicillium notatum." Arq. Inst. biol., Sao Paulo 16:307-314.

Meyer, J.R., 1946. Tentativas para separação da substância antineoplásica que se forma nos líquidos de cultura de "Penicillium notatum." Arq. Inst. biol., São Paulo 17:163-173.

Reggianini, O. and Brillanti, F., 1947. Prove biologiche sull' azione della penicillina crude (crude filtrate). Boll. Soc. ital. biol. sper. 23:19-20.

deRopp, R.S., 1946. Penicillin as a plant hormone. Nature 158:555.

Scudi, J.V. and Jelinek, V.C., 1944. On the inhibition of urease by penicillin. Science 100:312-313.

Smolens, J.; McAleer, D.S. and McLaren, C.S., 1947. Antidotal properties of crude Penicillium notatum filtrate. Proc. Soc. Exper. Biol. & Med. 65:172-175.

Taormina, A., 1946. Richerche sui caratteri fisici e farmacologici dei brodi filtrati ottenuti da culture di Penicillium notatum invecchiate. Boll. Soc. ital. biol. sper. 22:897-898.

Vargas, J.J. and Escubós, J.M., 1945. Estudio experimental sobre algunos aspectos de la acción de la ureasa y su inhibición por la Penicilina. Rev. espan. fisiol. 1:50-57.

Waksman, S.A., 1945. Microbial antagonisms and antibiotic substances. Commonwealth Fund. New York.

Welch, H.; Randall, W.A. and Price, C.W., 1947. Amorphous vs. crystalline penicillin. Mimeo.

MUSTARDS

Anslow, W.P., Jr.; Karnofsky, D.A.; Jager, B.V. and Smith, H.W., 1947. The toxicity and pharmacological action of the nitrogen mustards and certain related compounds. J. Pharmacol. & Exper. Therap. 91:224-235.

ApThomas, M.I.R. and Cullumbine, H., 1947. Nitrogen mustards in Hodgkin's disease; report of 21 cases and 4 of other reticuloses. Lancet 252:899-901.

Auerbach, C., 1947. The induction by mustard gas of chromosomal instabilities in Drosophila melanogaster. Proc. Roy. Soc. Edinburgh B62:307-320.

Auerbach, C. and Robson, J.M., 1947 a. The production of mutations by chemical substances. Proc. Roy. Soc. Edinburgh B62:271-283.

Auerbach, C. and Robson, J.M., 1947 b. Tests of chemical substances for mutagenic action. Proc. Roy. Soc. Edinburgh B62:284-291.

Barron, E.S.G.; Seegmiller, J.E.; Mendes, E.G. and Narahara, H.T., 1948. The effect of nitrogen mustards on the respiration and fertilization of sea urchin sperm and eggs. Biol. Bull. 94:267-274.

Bass, A.D. and Freeman, M.L.H., 1946. Response of certain mouse tumors to bis (β-chloroethyl) sulfide (mustard gas). J. Natl. Cancer Inst. 7:171-176.

Bodenstein, D., 1947 a. The effect of nitrogen mustards on proliferating embryonic tissues. Cancer Research 7:49-50.

Bodenstein, D., 1947 b. The effects of nitrogen mustards on embryonic amphibian development. Part 1. Ectodermal effects. J. Exper. Zool. 104:311-341.

Boyland, E., 1947. Cytological effects of nitrogen mustards. A.A.A.S. Cancer Conference, Aug. 13, 1947. To be published.

Boyland, E.; Koller, P.C. and Warwick, O.H., 1947. The effects of chloroethylamines on tumors. 4th Internat. Cancer Research Cong. Abstracts p.89.

Drews, M., 1939. Untersuchungen über die klinisch nachweisbare Resorptivwirkung bei Hautschädigungen durch Dichlorodiäthylsulfid bei der Katze. Ztschr. f. d. ges. exper. Med. 105:29-45.

Eisenstark, A. and Clark, G.L., 1947. Electron micrographs of x-ray-treated Escherichia coli cells. Science 105:553-555.

Erslev, A.J.; Burchenal, J.H.; Karnofsky, D.A.; Craver, L.F. and Rhoads, C.P., 1947. The clinical evaluation of nitrogen mustard derivatives in leukemia. 4th Internat. Cancer Research Congr. Abstracts p.55.

Fell, H.B. and Allsopp, C.B., 1948 a. The action of mustard gas (ββ'-dichlorodiethylsulphide) on living cells in vitro. Cancer Research 8:145-161.

Fell, H.B. and Allsopp, C.B., 1948 b. The effect of repeated applications of minute quantities of mustard gas (ββ'-dichlorodiethylsulphide) on the skin of mice. Cancer Research 8:177-181.

Flury, F. and Wieland, H., 1921. Über Kampfgasvergiftungen. VII. Die pharmakologische Wirkung des Dichloräthylsulfids. Ztschr. f. d. ges. exper. Med. 13:367-483.

Friedenwald, J.S. and Buschke, W., 1948. Nuclear fragmentation produced by mustard and nitrogen mustards in the corneal epithelium. Bull. Johns Hopkins Hosp. 82:161-177.

Friedenwald, J.S.; Buschke, W. and Scholz, R.O., 1948. Effects of mustard and nitrogen mustard on mitotic and would healing activities of the corneal epithelium. Bull. Johns Hopkins Hosp. 82:148-160.

Gillette, Roy and Bodenstein, D., 1946. Specific developmental inhibitions produced in amphibian embryos by a nitrogen mustard compound. J. Exper. Zool. 103:1-32.

Gilman, A. and Philips, F.S., 1946. The biological actions and therapeutic applications of the β-chloroethyl amines and sulfides. Science 103:409-415.

Goldfeder, Anna., 1945. Further studies on the relation between radiation effects, cell viability, and induced resistance to malignant growth. Radiology 44:283-292.

Golumbic, C.; Fruton, J.S. and Bergmann, M., 1946. Chemical reactions of the nitrogen mustard gases. I. The transformations of methyl-bis (β-chloroethyl) amine in water. J. Org. Chem. 11:518-535.

Goodman, L.S.; Wintrobe, M.M.; Dameshek, W.; Goodman, M.J. and others, 1946. Nitrogen mustard therapy. J. A. M. A. 132:126-132.

Graef, I.; Karnofsky, D.A.; Jager, V.B.; Krichesky, B. and Smith, H.W., 1948. The clinical and pathologic effects of the nitrogen and sulfur mustards in laboratory animals. Am. J. Path. 24:1-47.

Graef, I.; Karnofsky, D.A. and Smith, H.W., 1946. The clinical and pathologic effects of the vesicant nitrogen and sulfur mustards. Federation Proc. 5:221-222.

Hawkins, J.W. and Farber, S., 1947. The action of nitrogen mustard on certain tumors in children. 4th Internat. Cancer Research Congr. Abstracts p.56.

Hofmeyr, H.O., 1947. Clinical experiences with nitrogen mustard in Hodgkin's disease. South African M. J. 21:195-198.

Horowitz, N.H.; Houlahan, M.B.; Hungate, M.G. and Wright, B., 1946. Mustard gas mutations in Neurospora. Science 104:233-234.

Hutchens, J.O.; Podolsky, B. and McMahon, T.M., 1948. Effects of tris-(β-chloroethyl)-amine on respiration, carbohydrate and protein synthesis, and cell division in Chilomonas. Federation Proc. 7:59.

Jacobson, L.O.; Spurr, C.L.; Barron, E.S.G.; Smith, T. and others, 1946. Nitrogen mustard therapy. J. A. M. A. 132:263-271.

Karnofsky, D.A.; Burchenal, J.H.; Ormsbee, R.A.; Cornman, I. and Rhoads, C.P., 1947. Experimental observations on the use of the nitrogen mustards in the treatment of neoplastic disease. American Association for the Advancement of Science. Approaches to Tumor Chemotherapy, The Association. Washington, D.C. p.293-305.

Karnofsky, D.A.; Craver, L.F.; Rhoads, C.P.; Abels, J.C. and McElroy, M.E., 1947. An evaluation of methyl-bis (β-chloroethyl)amine hydrochloride and tris(β-chloroethyl)amine hydrochloride (nitrogen mustards) in the treatment of lymphomas, leukemia and allied diseases. American Association for the Advancement of Science, Approaches to Tumor Chemotherapy. The Association. Washington, D.C. p.319-337.

Kindred, J.E., 1946. A quantitative study of the hemopoietic organs of albino rats following intravenous injection of mustard and nitrogen mustards. Anat. Rec. 94:474.

Kindred, J.E., 1947. Histologic changes occurring in the hemopoietic organs of albino rats after single injections of 2-chloroethyl vesicants. Arch. Path. 43:253-295.

Kinsey, V.E. and Grant, W.M., 1947 a. Action of mustard gas and other poisons on yeast cells. I. Effect of mustard gas on the rate of cell division. J. Cell. & Comp. Physiol. 29:51-64.

Kinsey, V.E. and Grant, W.M., 1947 b. Action of mustard gas and other poisons on yeast cells. II. Study of the relationship between inhibition of carbohydrate metabolism and inhibition of growth by various poisons, and effects of other toxic agents on yeast. J. Cell. & Comp. Physiol. 30:31-42.

Koller, P.C., 1947. The behavior of the tumour cell under the influence of irradiation and chemicals. 4th Internat. Cancer Research Congr. Abstracts p.142.

Krumbhaar, E.B., 1919. Role of the blood and the bone marrow in certain forms of gas poisoning. I. Peripheral blood changes and their significance. J. A. M. A. 72:39-41.

Krumbhaar, E.B. and Krumbhaar, H.D., 1919. The blood and bone marrow in yellow cross gas (mustard gas) poisoning. Changes produced in the bone marrow of fatal cases. J. M. Research 40:497-507.

Lillie, R.S.; Clowes, G.H.A. and Chambers, R., 1919 a. Preliminary report of experiments on the action of dichloroethylsulfide (mustard gas) on the cells of marine organisms. Science 49:382-385.

Lillie, R.S.; Clowes, G.H.A. and Chambers, R., 1919 b. On the penetration of dichloroethylsulphide (mustard gas) into marine organisms, and the mechanism of its destructive action on protoplasm. J. Pharmacol. & Exper. Therap. 14:75-120.

Marshak, A., 1946. Effect of mustard gas on mitosis and P32 uptake in regenerating liver. Proc. Soc. Exper. Biol. & Med. 63:118-120.

Maier, G., 1938. Experimentelle Untersuchungen zur Frage der Allgemein-Schädigungen durch Dichlordiäthylsulfid (Gelbkreuzkampfstoff). Ztschr. f. d. ges. exper. Med. 103:458-478.

Meier, R. and Schär, B., 1947. Différentiation de l'action antimitotique sur la cellule animale, in vitro. Experientia 3:358-366.

Muntsch, O., 1934. Die Blutveränderungen bei Kampfgaserkrankungen als diagnostisches Hilfsmittel. Klin. Wchnschr. 13:482-485; 529-532.

Pappenheimer, A.M. and Vance, M., 1920. The effects of intravenous injections of dichloroethylsulfide in rabbits, with special reference to its leucotoxic action. J. Exper. Med. 31:71-94.

Philips, F.S. and Gilman, A., 1947. The relation between the chemical constitution and biological action of the nitrogen mustards. American Association for the Advancement of Science. Approaches to Tumor Chemotherapy. The Association. p.285-292.

Rhoads, C.P., 1946. Nitrogen mustards in the treatment of neoplastic disease. J. A. M. A. 131:656-658.

Richters, C.E., 1932. Die Tiere in chemischen Krieg. Verlags Buchhandlung von Richard Schoetz, Berlin.

Schrek, R., 1947. A comparison of the reaction of cells to nitrogen mustard and x-rays. 4th Internat. Cancer Research Congr. Abstracts p.54.

Slizynska, H. and Slizynski, B.M., 1947. Genetical and cytological studies of lethals induced by chemical treatment in Drosophila melanogaster. Proc. Roy. Soc. Edinburgh B62:234-242.

Spurr, C.L., 1947. The clinical application of a nitrogen mustard compound methyl bis (β chloroethyl) amine to the treatment of neoplastic disorders of the hemopoietic system. A.A.A.S. Cancer Conference, Aug. 13, 1947. To be published.

Spurr, C.L.; Jacobson, L.O.; Smith, T.R. and Barron, E.S.G., 1947. The clinical application of a nitrogen mustard compound methyl bis (β chloroethyl) amine to the treatment of neoplastic disorders of the hemopoietic system. Cancer Research 7:51-52.

Taffel, M., 1947. Experiences in the treatment of neoplastic disease with nitrogen mustard. Yale J. Biol. & Med. 19:971-977.

Warthin, A.S. and Weller, C.V., 1919. The medical aspect of mustard gas poisoning. C.V. Mosby, St. Louis.

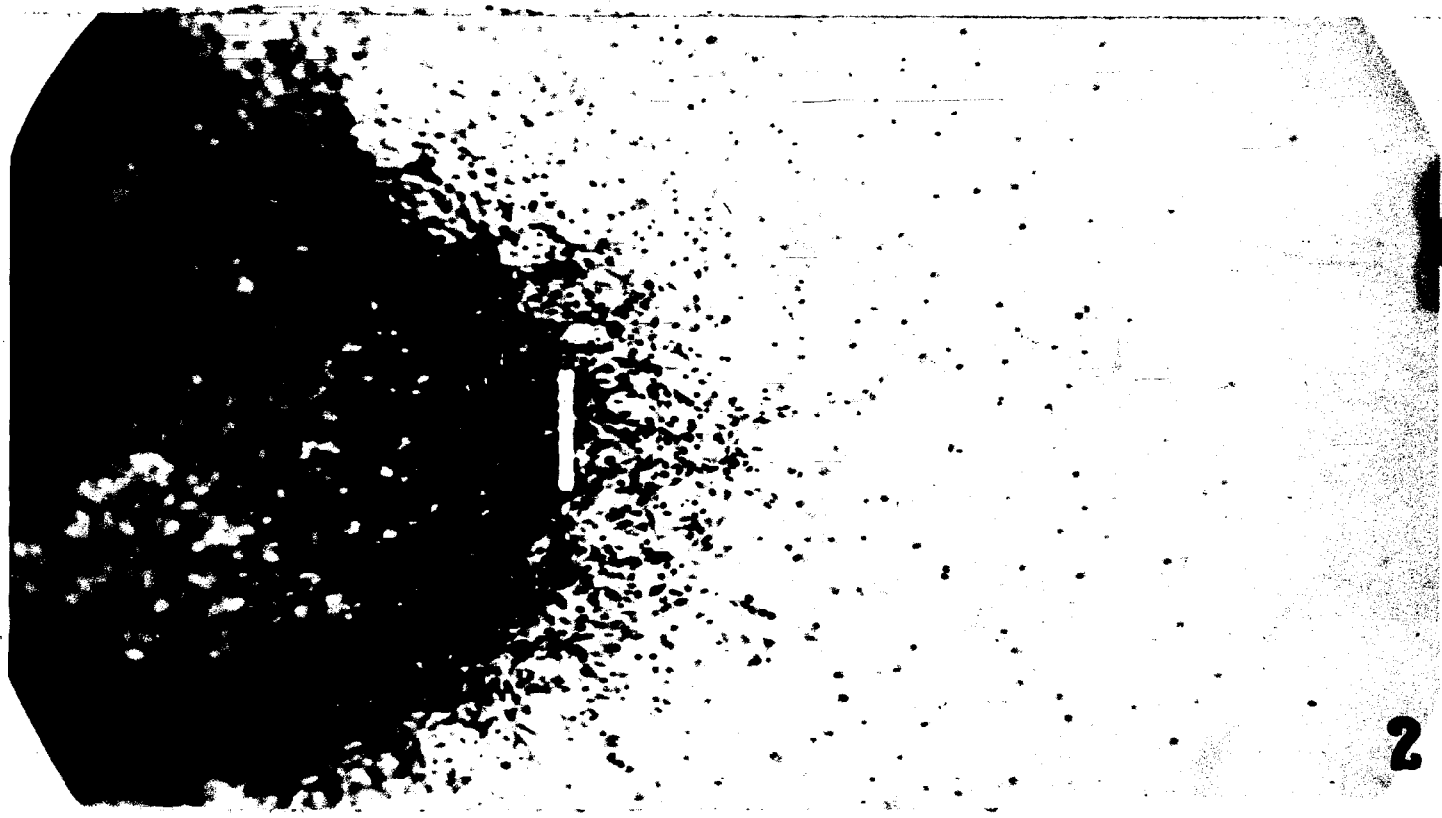
Wilkinson, J.F. and Fletcher, F., 1947. Effect of β-chloroethylamine hydrochlorides in leukemia, Hodgkin's disease and polycythaemia vera; report of eighteen cases. Lancet 253:540-545.

EXPLANATION OF FIGURES

Figs. 1 and 2. Muscle (Fig. 1) and sarcoma 132 (Fig. 2) growing in the same tube after 12 days' exposure to penicillin. The vigorous growth of the muscle forms a migration zone as broad as the diameter of the original explant, whereas the sarcoma shows only a sparse fringe of cells and scattered, rounded, moribund cells. A white bar indicates the edge of the explant. X 45.

Materials for Figs. 1 - 6 fixed in Bouin's and stained in hematoxylin.

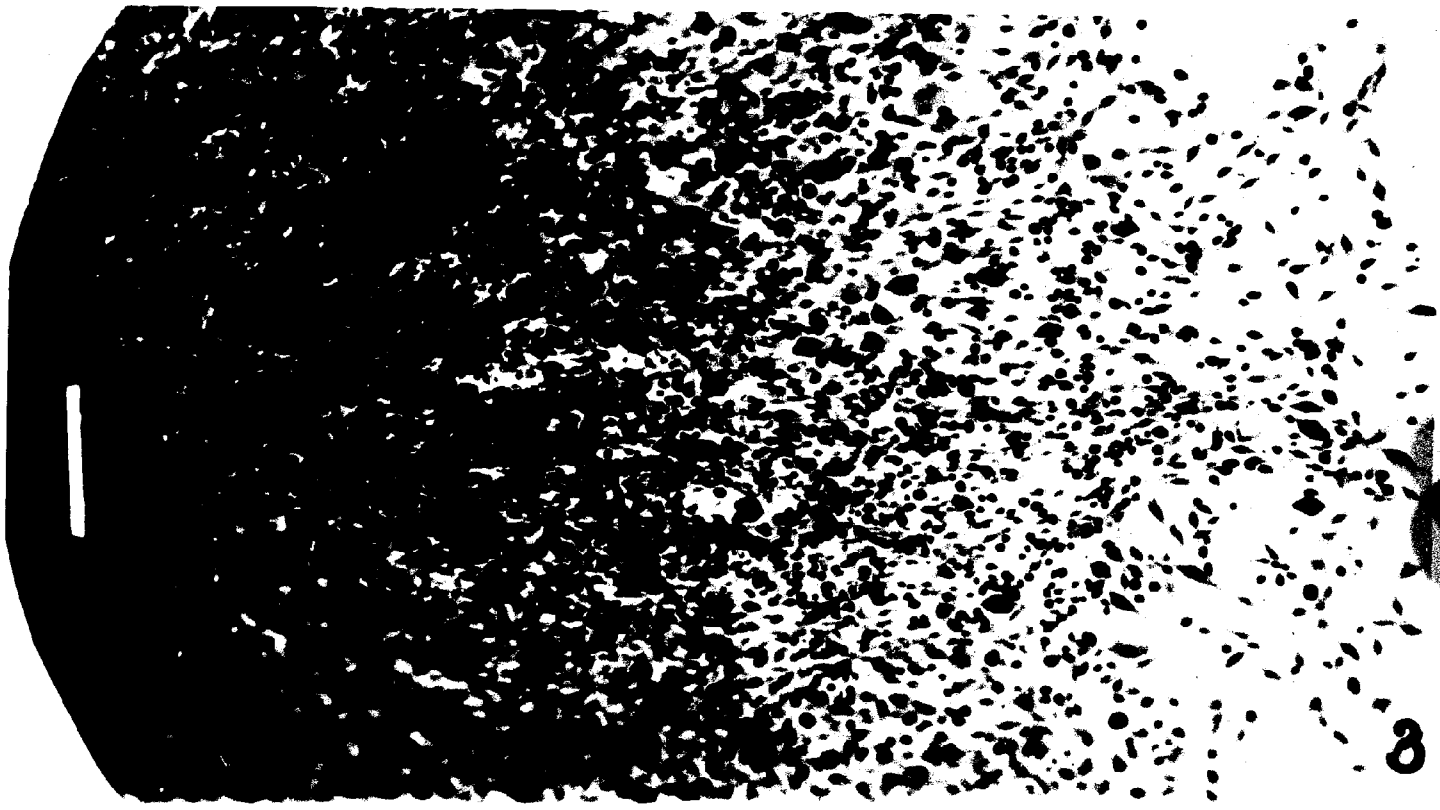
1



2

Fig. 3. Sarcoma 132 untreated. The 5 days' growth is equal to the 12 days' growth of the muscle in Fig. 1. X 45.

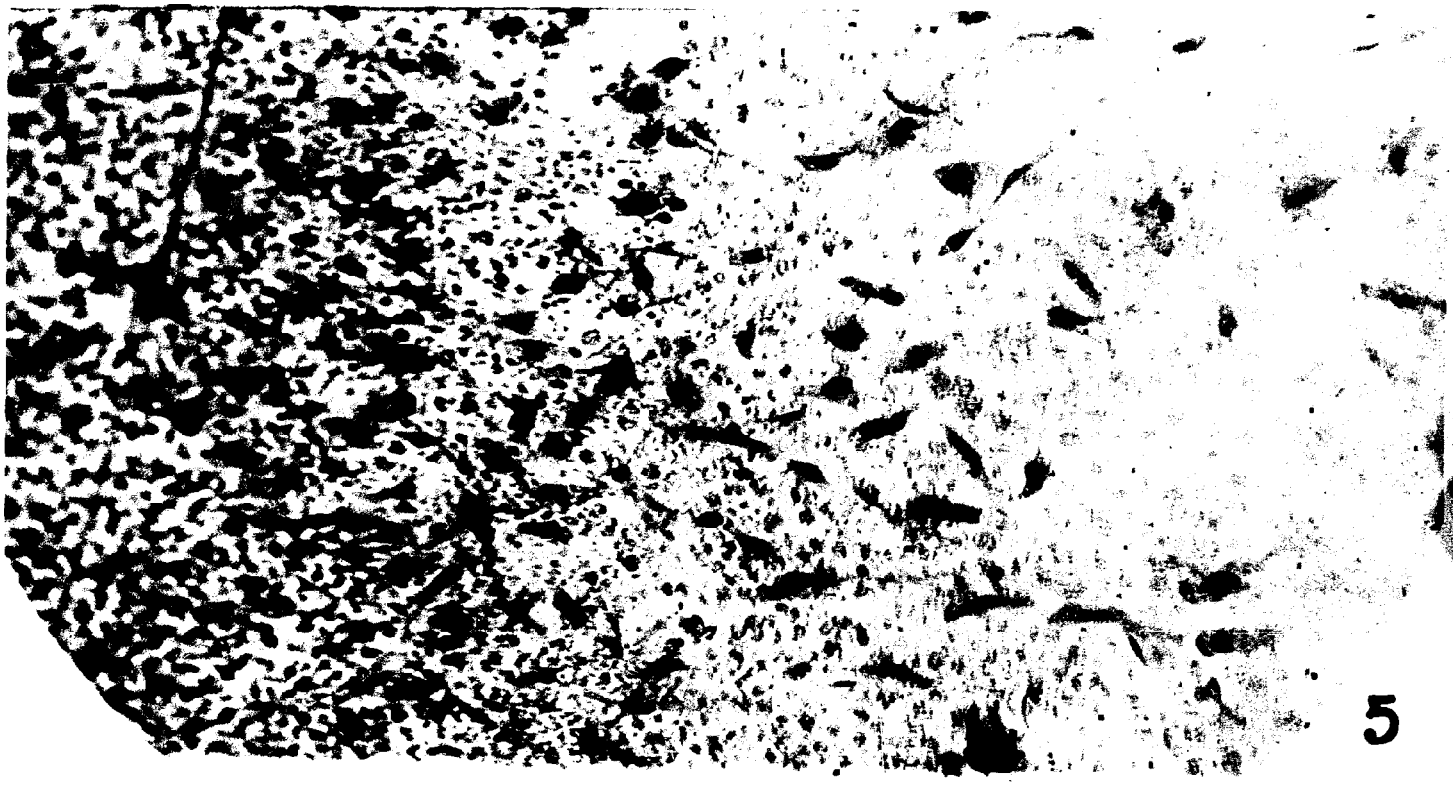
Fig. 4. Muscle (Fig. 4) and sarcoma 11 (Fig. 5) growing in the same tube after six days' exposure to penicillin followed by 2 days in normal medium. The cells in the muscle migration zone are the normal fibroblastic type. X 100.


3


4

Fig. 5. Sarcoma growing in same tube as the muscle fibroblasts shown in Fig. 4. The sarcoma migration zone is composed only of deformed cells and debris of disintegrated cells. The effect was graded only as "marked damage" inasmuch as some apparently viable cells remain, but five sister colonies from the same tube, implanted into one rat, failed to produce a tumor. X 100.

Fig. 6. Untreated cells of tumor 132. Same explant as in Fig. 3. X 100.


5

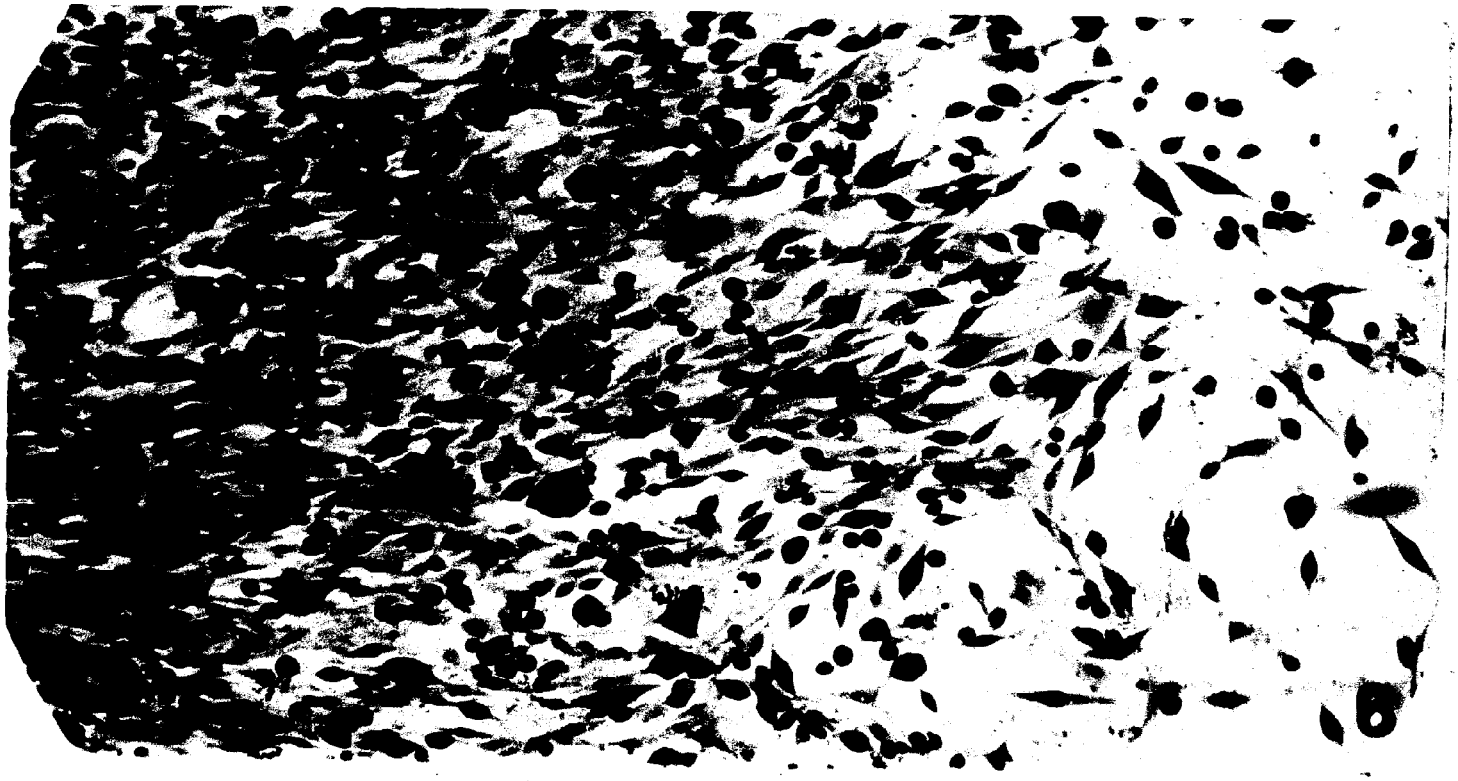
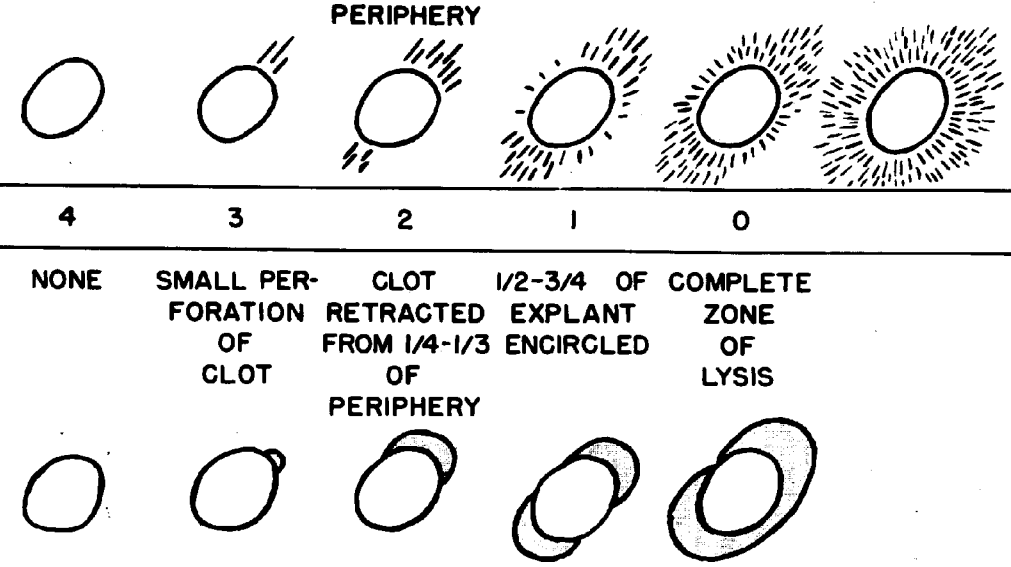

6

Fig. 7. Criteria for grading inhibition and damage of tissue cultures. The grades for lysis are inverted since there is less lysis with greater inhibition.

GRADING OF GROWTH AND LYSIS IN TISSUE CULTURES

	GRADE	0	1	2	3	4	5
GROWTH	EXTENT OF GROWTH	NONE	FEW MIGRATING CELLS	CELLS EMERGING ALONG 1/4-1/3 OF PERIPHERY	1/3-3/4 OF EXPLANT ENCIRCLED	COMPLETE ZONE OF GROWTH	GROWTH ZONE AS WIDE AS EXPLANT
	GRADE OF INHIBITION	4	3	2	1	0	
LYSIS	EXTENT OF LYSIS	NONE	SMALL PERFORATION OF CLOT	CLOT RETRACTED FROM 1/4-1/3 OF PERIPHERY	1/2-3/4 OF EXPLANT ENCIRCLED	COMPLETE ZONE OF LYSIS	



GRADING OF GRANULARITY AND ROUNDING IN TISSUE CULTURES

	0	1	2	3	4
GRANULATION					
ROUNDING					

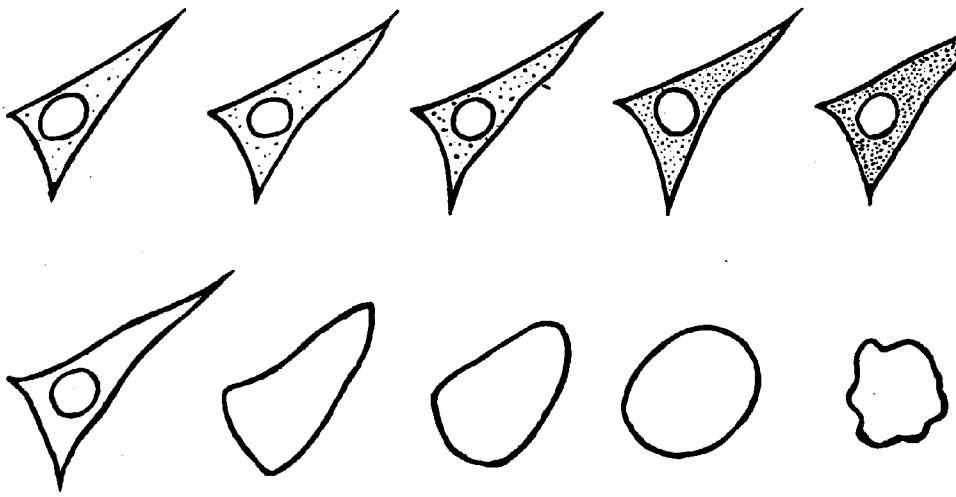


FIGURE 7

Fig. 8. Formulae of Nitrogen Mustards

I Methyl bis-(β-chloroethyl)-amine, or HN2.

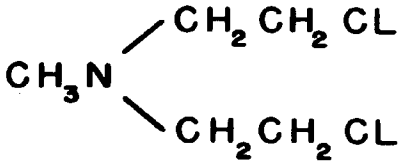
II Tris-(β-chloroethyl)-amine, or HN3.

III Bis-[bis-(β-chloroethyl)-amino]-ethane.

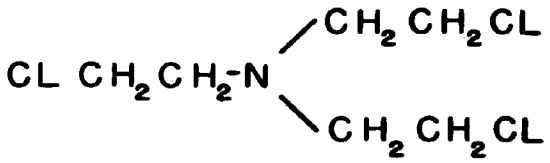
IV 1,3-bis-[bis-(β-chloroethyl)-amino]-propane.

V 2-chloro-1,3-bis-[bis-(β-chloroethyl)-amino]-propane.

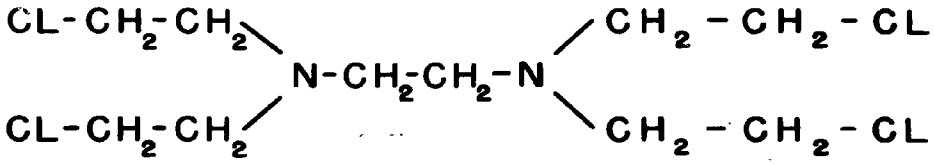
These were used as the hydrochlorides (I & II) or dihydrochlorides (III, IV, V).



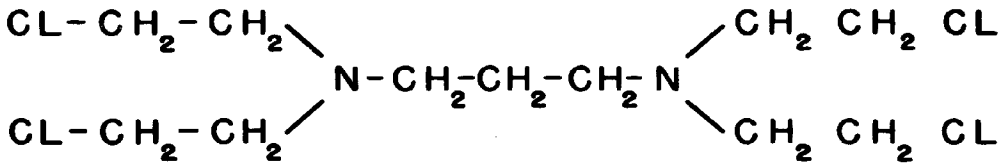
I



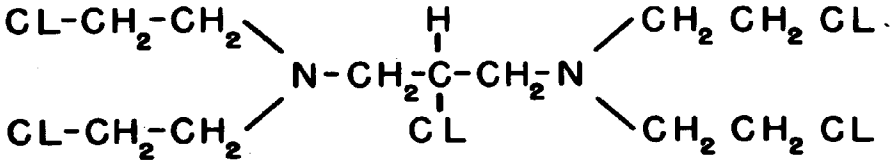
II



III



IV



V

FIGURE 8

Figures 9 - 20

Normal fetal mouse skin, mouse carcinoma E060 and sarcoma 202L were grown in normal medium for 48 hours, then exposed to 10, 20 and 40 mg/L of HN3 (compound II) for one day. Embryonic chick heart was exposed to 100 mg/L. They were returned to normal medium and fixed 5 days after termination of the exposure. Fixative: 10% formalin; stain: Harris hemalum. X 200 in figures 14 - 17 and 20; X 100 in others.

Figs. 9 - 12

Fig. 9. Mouse fibroblasts grown to gigantic proportions following exposure to 10 mg/L.

Fig. 10. Normal mouse fibroblasts, the largest to be found in the control culture.

Fig. 11. Typical population of enlarged cells following exposure to 40 mg/L.

Fig. 12. Typical population of untreated mouse fibroblasts.

9

10

11

12

Fig. 13. Tracings of normal and exposed mouse fibroblasts on centimeter coordinate paper.
A: The largest cells to be found in the control tube; average area 600 sq. μ. B: Representative sample of cells exposed to 20 mg/L; average area 1700 sq. μ.
C: Representative sample of cells exposed to 40 mg/L; average area 2800 sq. μ.

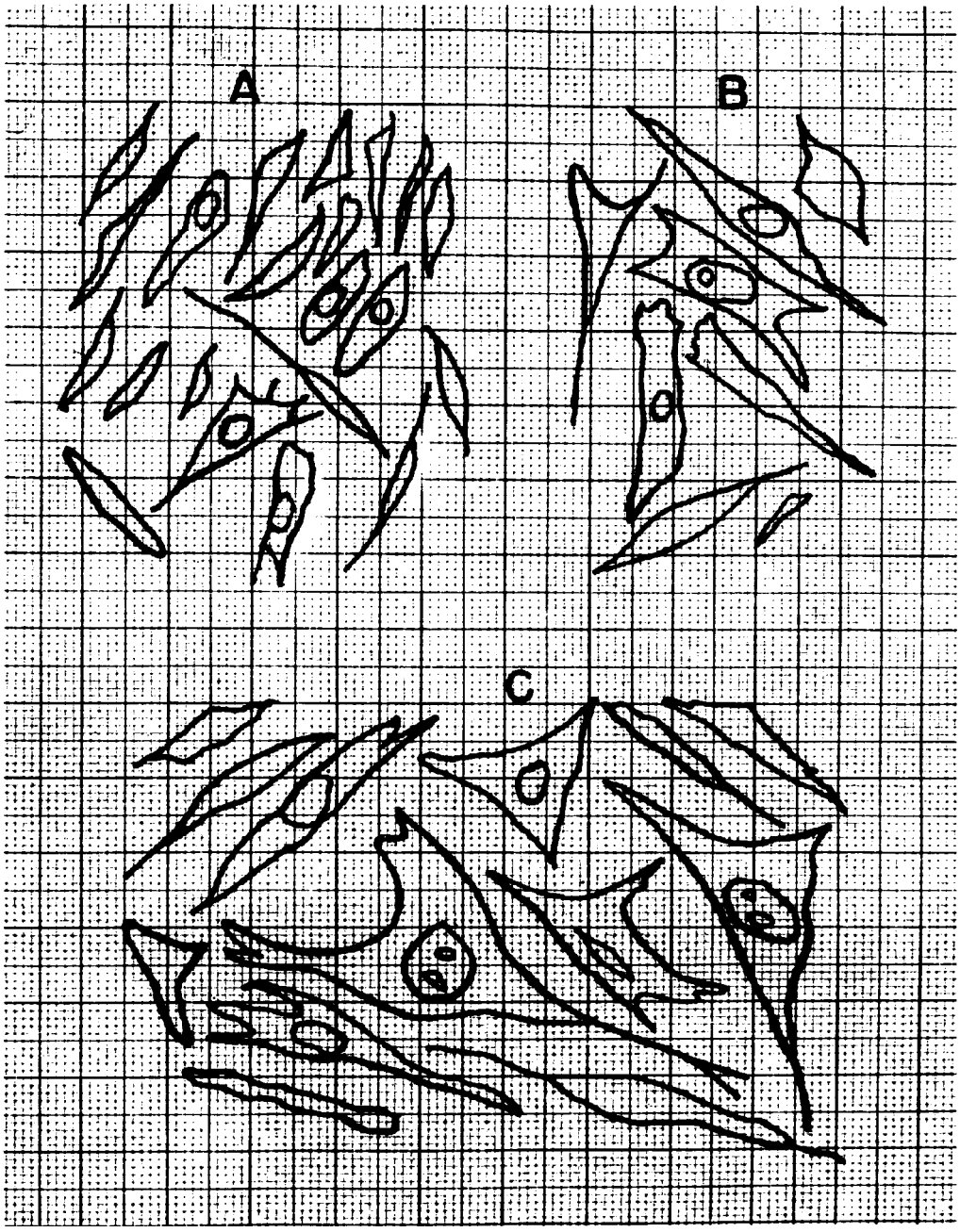


FIGURE 13

Fig. 14. Sarcoma 202L after exposure to 20 mg/L.

Fig. 15. Largest cells (note binucleation) in the untreated culture of 202L.

Fig. 16. Carcinoma E060 after exposure to 40 mg/L. As in any epithelium the cell boundaries are indistinct. Compare size of nuclei.

Fig. 17. Untreated E060. Cell rounding up for division in lower right.

14

15

16

17

Fig. 18. Giant fibroblast from chick heart following exposure to 100 mg/L. The nucleus and nucleoli are clearly visible in the center. The thin cytoplasm extends across the middle third of the photograph.

Fig. 19. Untreated chick fibroblasts.

Fig. 20. Tripolar mitosis in chick fibroblast exposed to 100 mg/L.

18

19

20