Biochemical Pharmacology, 1960, Vol. 5, pp. 53–63. Pergamon Press Ltd., Printed in Great Britain.

THE CYTOCIDAL ACTION OF MITOTIC POISONS ON LYMPHOCYTES *IN VITRO*

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(*Received* 10 *May* 1960)

Abstract—Rat lymph nodes were maintained *in vitro* by an organ culture technique. The lymphocytocidal effects of the following substances were investigated: nitrogen mustard, acriflavine, quinone, hydroquinone, rotenone, cortisone, colchicine, podophyllotoxin, chelidonine, arsenite, stilboestrol, selenite, diphenylhydantoin, cyanide. This list includes all the substances known to kill lymphocytes *in vitro* at 10^{-4}M and most of the mitotic poisons shown (by others) to act on fibroblasts *in vitro* at 10^{-5}M. For each substance the LC at 50–48 hr (concentration which kills 50 per cent of the lymphocytes in 48 hr) was determined from the dose–effect curve.

In the cases of nitrogen mustard, acriflavine, rotenone, colchicine, arsenite and stilboestrol, the LC_{50} for lymphocytes was found to be about the same as the concentration required for mitotic poisoning of fibroblasts. But the mitotic poisons quinone–hydroquinone and chelidonine were not particularly poisonous to lymphocytes, and the specific lymphocyte poisons diphenylhydantoin and selenite are not mitotic poisons. The results do not encourage any simple idea of a single biochemical lesion common to lymphocyte and mitotic poisoning. Such correlation as exists may simply reflect the fact that mitosis is the cell process and the lymphocyte the cell type most vulnerable to adverse influences.

Rotenone is by far the most potent lymphocyte poison so far discovered; in 10^{-9}M concentration it kills at least one in every five cells.

INTRODUCTION

A VERY wide variety of chemical substances,[1] including distilled water,[2] have now been shown to inhibit or derange the process of cell division. Moreover, mitosis can be affected in several different ways. The term "mitotic poison" has thus come to be so broadly and variously used as to lose much of its former significance. Recent attempts to redefine the term in a more meaningful way have only revealed considerable differences of opinion.[1] So it remains the first duty of an author to explain what he means by "mitotic poisoning".

The mitotic poisons considered in this paper are those powerful poisons which specifically either prevent or arrest mitosis in a wide variety of animal cells. More precisely, they all inhibit mitosis in fibroblast cultures *in vitro* at concentrations of 10^{-5} M or less (Table 1, column E), with no other observable effect on the cells (except that a few cause chromosome breakage). Most of them have been shown to inhibit the mitosis of sea urchin eggs at similarly low concentrations (Table 1, column D). The various metabolic inhibitors of respiration, glycolysis, phosphorylation, etc., are excluded from consideration for they only act at concentrations of about 10^{-4} M or more and they affect other cell processes as well. The actions of the powerful specific mitotic poisons cannot yet be explained in chemical terms.

TABLE 1

(A1 = chromosome poisons, A2 = spindle poisons, A3 = not mitotic poisons. Literature references in brackets. In some cases the figures have been calculated from data given in the publications cited. Column C gives the minimum dose reported to cause obvious pyknosis of lymphocytes in lymph nodes or thymus within 12 hr, as seen in histological sections. Column E gives, for the chromosome poisons—the minimum concentration reported to cause a marked (usually 50 per cent) reduction in the mitotic index, for the spindle poisons—the minimum concentration reported to cause an obvious accumulation of arrested metaphases.

	B Lethal dose (rat) (mg/kg) (s.c. = subcutaneous, i.v. = intravenous, i.p. = intraperitoneal)			C Dose to kill lymphocytes in the lymphoid tissues (rat or mouse) (mg/kg)		D Concentration for mitotic poisoning of sea-urchin eggs		E Concentration for mitotic poisoning of fibroblasts *in vitro*		F LC at 50–48 hr for lymphocytes *in vitro*
A1										
Nitrogen Mustard	1·1	i.v.	(10)	1·0	(24)	10^{-3}M	(31)	10^{-5} M	(37)	$3{\cdot}7 \times 10^{-6}$ M
	1·9	s.c.	(10)							
Acriflavine	33·0	s.c.	(11)	25·0	(25)			$2{\cdot}5 \times 10^{-6}$ M	(38)	10^{-5} M
								10^{-5} M	(37, 39)	
Quinone	25·0	i.v.	(12)			7×10^{-6} M	(32)	10^{-9} M	(37)	$1{\cdot}9 \times 10^{-4}$ M
Hydroquinone	115·0	i.v.	(12)	125·0	(3)	5×10^{-6} M	(32)	6×10^{-9} M	(40)	$2{\cdot}1 \times 10^{-4}$ M
Rotenone	5·0	i.p.	(13)	10·0	(26)	5×10^{-9} M	(33)	$2{\cdot}5 \times 10^{-8}$ M	(41)	$2{\cdot}7 \times 10^{-9}$ M
	6·0	i.v.	(14)							
Cortisone				12·5	(27)	3×10^{-4} M	(34)	5×10^{-7} M	(42)	$3{\cdot}5 \times 10^{-5}$ M
A2										
Colchicine	1·7	i.v.	(15)	2·0	(28)	10^{-5} M	(35)	$2{\cdot}5 \times 10^{-8}$ M	(39)	$2{\cdot}3 \times 10^{-7}$ M
	4·0	s.c.	(16)	1·3	(29)			5×10^{-8} M	(38)	
Podophyllotoxin	8·0	s.c.	(17)	8·0	(17)	$1{\cdot}5 \times 10^{-6}$ M	(36)	10^{-5} M	(43)	10^{-7} M
Chelidonine	300·0	s.c.	(18)					$2{\cdot}5 \times 10^{-6}$ M	(44)	
Na arsenite	10·0	i.p.	(19)	2·3	(30)			$3{\cdot}4 \times 10^{-6}$ M	(45)	$2{\cdot}2 \times 10^{-6}$ M
Stilboestrol	500·0	s.c.	(20)			2×10^{-6} M	(32)	$1{\cdot}5 \times 10^{-5}$ M	(46)	$1{\cdot}2 \times 10^{-5}$ M
								4×10^{-5} M	(44)	
A3										
Na selenite	3·0	i.v.	(21)	5·0	(3)			10^{-3} M	(47)	$4{\cdot}6 \times 10^{-6}$ M
	7·5	i.p.	(19)							
Diphenylhydantoin	280·0	i.p.	(22)					$>10^{-4}$ M	(48)	$6{\cdot}3 \times 10^{-5}$ M
K cyanide	2·5	i.v.	(23)					5×10^{-3} M	(47)	$1{\cdot}6 \times 10^{-4}$ M

Following Dustin[3], these nitotic poisons can be divided into two groups, "chromosome poisons" and "spindle poisons". The chromosome poisons, such as acriflavine and nitrogen mustard, affect the cells before division, preventing the onset of mitosis, and in many cases breaking the chromosomes as well. It seems unlikely, however, that chromosome breakage is the cause of the mitotic inhibition; recent work suggests that these are separate and unrelated effects.[4] The spindle poisons, such as colchicine and arsenic, affect cells during division, destroying the spindle and arresting the cells in metaphase. The chromosome damage in the one case or the spindle damage in the other may be so great that the cells ultimately die.

The division of mitotic poisons into these two groups has often been criticized because some poisons can exhibit both actions, depending on the concentration. But even so it seems legitimate to maintain a distinction between the two types of mitotic poisoning and the belief that this reflects two different points of chemical attack on the mitotic process. In Table 1 (column A) the mitotic poisons have been allocated to one or other of these two groups (A1, A2) on the basis of the action which they exhibit at lowest concentration.

So far, it has been implied that these poisons affect only cells in division or about to divide, but this is not the whole truth. Many of them have an additional and totally different action. Injected into animals in antimitotic doses they selectively kill the lymphocytes in all the lymphoid tissues. The cells in question are nearly all small lymphocytes which never divide (except in the thymus cortex in some species), so the question of mitotic poisoning does not arise. Small doses of ionizing radiation also inhibit mitosis in general and kill small lymphocytes in particular.

The purpose of the present work was to explore more fully this apparent parallelism between mitotic inhibition and lymphocyte killing. Using rat lymph nodes maintained *in vitro*, the various mitotic poisons were added to the culture medium and in each case the concentration range required to kill the lymphocytes was accurately determined. The concentrations needed to kill lymphocytes *in vitro* were then compared with the concentrations which have been shown to inhibit mitosis in fibroblast cultures *in vitro*. Results obtained with sodium selenite and diphenylhydantoin are also included, for comparative purposes. These two substances are specifically toxic to lymphocytes but they are not mitotic poisons, (as here defined) and they are put in column A3 in Table 1. Also included in A3 is cyanide as an example of a non-specific poison.

Investigations of this sort can be carried out only *in vitro*, for only so can a direct effect of the poison be demonstrated and the concentration applied be exactly known. In the whole animal the concentration of poison reaching the cells and its length of stay are unknown and in the particular case of lymphocytes these cells may also be killed indirectly by the pituitary-adrenal stress reaction. For these reasons, results obtained by injecting mitotic poisons into animals (Table 1, column C), though suggestive, are unacceptable for quantitative consideration.

METHODS

Suspensions of lymphocytes do not survive satisfactorily *in vitro* and it has been found better to use lymph nodes from small animals (rats) maintained *in vitro* by an organ culture technique.[5] In these lymph-node cultures the lymphocytes remain alive and healthy for about a week,[5] though there is no production of new cells by mitosis of precursors. More than 99 per cent of the lymphocytes are mature small lymphocytes.[6]

This survival time is ample for the study of chemical toxicity which was done by counting the percentage of dead (pyknotic) lymphocytes at time intervals (5–48 hr) after addition of the poison.

This method has already been used to study the cytocidal action of X-rays,[7] cortisone[8] and barbiturates[9] on lymphocytes *in vitro*. In the present work the method previously described in this *Journal*[9] was followed exactly, using the lumbar and sacral lymph nodes of 4-week-old rats. It only remains to describe how the poisons were made up and added.

The nitrogen mustard used was Mustine Hydrochloride BPC (Boots Pure Drug Co.). Podophyllotoxin and Chelidonine were obtained from L. Light and Co. Sodium-5 : 5-diphenylhydantoinate ("epanutin") was obtained from Parke, Davis and Co. Cortisone (free alcohol) and rotenone (material 51R 3753) were obtained from Merck and Co. The rotenone was about 99 per cent pure; most commercial samples are very impure. All the other substances were obtained from British Drug Houses.

"Initial" solutions were made up not more than an hour before addition to the cultures, at a concentration of 10^{-1} M for the weak poisons and 10^{-2} or 10^{-3} M for the stronger ones. Colchicine, acriflavine, sodium arsenite, sodium selenite and KCN were made up in 10 ml volumes of distilled water and the solutions sterilized by suction through a No. 5 porosity sintered glass filter. Nitrogen mustard was dissolved from a sterile ampoule in sterile distilled water and added to the cultures within 5 min. In the case of all the other substances, the required weight for 10 ml of solution was placed in a sterile graduated test tube and dissolved in 4 ml of ethanol, after which the volume was made up to 10 ml with sterile distilled water. The alcohol sterilized the solution and no filtration was necessary.

For the highest concentrations required in the culture medium the initial solution was added directly in the amount of 0·05 ml or 0·015 ml per 5 ml of medium. For lower concentrations the initial solution was first diluted 1 in 10, 1 in 100 or 1 in 1000 with sterile distilled water and then added in the amount of 0·05 or 0·015 ml per 5 ml of medium. The final concentration of ethanol in the medium never exceeded 0·4 per cent and control experiments[9] had shown that 10^{-1} M ethanol (0·46 per cent) was quite harmless.

In some cases the upper part of the effective concentration range could not be covered completely because of difficulty in solubility (cortisone, chelidonine) or cytological fixation effects (quinone, hydroquinone).

Some of the figures for cortisone,[8] colchicine[9] and cyanide[9] have been published previously in other contexts.

RESULTS

Fig. 1 shows that after addition of colchicine or rotenone, the percentage of dead lymphocytes in the culture (the "pyknotic count") increases more or less linearly with time for the first 48 hr. It has been assumed that this linear relationship also holds good for the other poisons, and 48 hr was chosen as a convenient (but arbitrary) end-point at which to measure the effect. The effects of different concentrations and different poisons were thereby compared. The results are given in Table 2, where the poisons are listed in descending order of lymphocyte toxicity, and the dose-effect curves are plotted in Fig. 2.

From these curves can be read off the LC at 50-48 hr for each poison (the concentration which kills 50 per cent of the lymphocytes in 48 hr). This, however, is not the exact lymphocyte LC_{50}, for by the time the cells were fixed and counted (48 hr) some of the dead lymphocytes had doubtless been removed by phagocytosis or autolysis, so the true LC_{50} must be somewhat higher than that read from these results. Nevertheless this qualified LC_{50} is a convenient yard stick with which to compare one poison with another. The LC_{50} figures are listed in column F of Table 1.

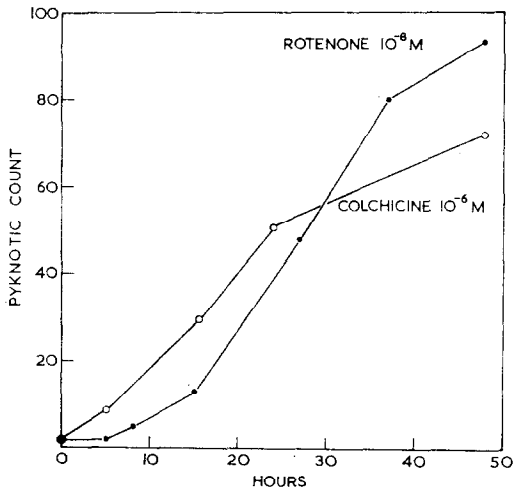

FIG. 1. Percentage of dead lymphocytes (pyknotic count) in lymph-node cultures at various time intervals after addition of colchicine or rotenone.

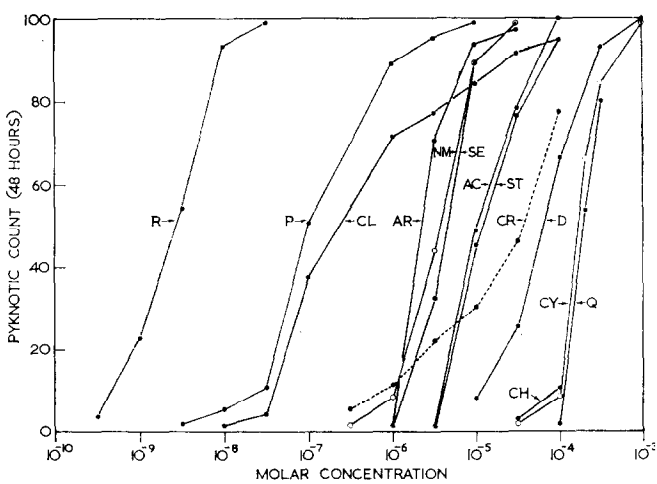

FIG. 2. Percentage of dead lymphocytes (pyknotic count) in lymph-node cultures 48 hr after addition of various concentrations of cell poisons: AC = acriflavine, AR = arsenite, CH = chelidonine, CL = colchicine, CR = cortisone, CY = cyanide, D = diphenylhydantoin, H = hydroquinone, NM = nitrogen mustard, P = podophyllotoxin, Q = quinone, R = rotenone, SE = selenite, ST = stilboestrol.

TABLE 2. PERCENTAGE OF DEAD LYMPHOCYTES (PYKNOTIC COUNT) IN LYMPH-NODE CULTURES 48 HR AFTER ADDING VARIOUS CONCENTRATIONS OF VARIOUS POISONS

(Each entry is the average value for from eighteen to twenty cultures. Control cultures (48 hr after no addition) = 1·2 per cent. F = cytological fixation. Percentage of reticulum cells dead indicated by: *<10, † = about 50, ‡ = nearly 100.)

Molar Concentration	3×10^{-10}	10^{-9}	3×10^{-9}	10^{-8}	3×10^{-8}	10^{-7}	3×10^{-7}	10^{-6}	3×10^{-6}	10^{-5}	3×10^{-5}	10^{-4}	2×10^{-4}	3×10^{-4}	10^{-3}
Rotenone	3·7*	22·7*	54·2*	93·4*	99·0†										
Podophyllotoxin			1·9*	5·4*	10·9*	50·4*		89·1*	95·2*	99·0†					
Colchicine				1·5*	4·3*	37·4*		71·9*	77·5*	84·5*	91·4†	94·6†			
Na arsenite								1·5*	70·9*	93·7*	97·2†				
N_2 mustard							1·7*	8·3*	44·1*	89·5*	99·0‡				
Na selenite								1·4*	32·7*	88·6*	100‡	F			
Acriflavine									1·3*	48·8*	78·5†	100†			F
Stilboestrol									1·2*	45·3*	76·7*	95·0‡			
Cortisone							5·4*	11·6*	22·1*	30·4*	46·1*	77·8*			
Diphenylhydantoin								2·0*		8·2*	25·5*	66·6*		93·1*	100‡
Chelidinine											2·6*	10·4*			
K cyanide											1·9*	8·5*	66·3†	84·6‡	99·1‡
Quinone												1·9*	53·8*	80·3†	F
Hydroquinone												2·5*	46·5*	80·4†	F

Table 2 shows also, by a system of *, †, ‡ scoring, the concentrations at which these various poisons killed the reticulum cells of the lymph node. Dead cells were recognized by nuclear pyknosis or karyolysis. There are always a few reticulum cells in the film preparations made from these cultures, enough to make this rough assessment but not enough for accurate counting. The results show that, except for cyanide which kills lymphocytes and reticulum cells about equally, all these poisons are selectively toxic to lymphocytes. In general, the concentration required to kill 50 per cent of the reticulum cells was at least ten times that needed to kill 50 per cent of the lymphocytes, and there was never any reticulum cell damage at the lymphocyte LC_{50} concentration.

Some of these poisons, in concentrations somewhat higher than those needed to kill lymphocytes, were found to act as histological fixatives. Acriflavine 10^{-3} M, hydroquinone 10^{-3} M and sodium selenite 10^{-4} M behaved in this way. In cultures exposed to these concentrations the lymphocytes and reticulum cells were all fixed, albeit rather poorly from a technical point of view, and there was of course no pyknosis. This fixative action was confirmed by fixing whole lymph nodes and thymus glands for 24 hr in corresponding solutions made up in isotonic saline, then embedding and cutting histological sections in the ordinary way. The histological structure was reasonably well preserved. Acriflavine is known to combine with and precipitate nucleic acids,[49] so its action is easily understood. Hydroquinone and selenite do not precipitate proteins or nucleic acids and their fixing action is difficult to explain. It may be recalled, however, that the fixing action of formaldehyde is similarly not understood.

DISCUSSION

The main object of this work was to find out if there is a universal parallelism between the antimitotic and the lymphotoxic effects of poisons acting in concentration of 10^{-5} M or less. In particular, is the relation sufficiently close to suggest a common underlying mode of action? The answer can be clearly seen in Fig. 3, and the two most important points emerging from the results are as follows.

(1) Virtually all the mitotic poisons known to be effective (on fibroblasts) at 10^{-5} M or less have been tested and all were found to be specifically toxic to lymphocytes at 10^{-5} M or less, except *quinone-hydroquinone* and *chelidonine*.

(2) In the course of other work (unpublished) we have investigated the toxicity of a very wide variety of chemicals to lymphocytes *in vitro* and all those exhibiting marked toxicity at 10^{-4} M or less are reported here in Table 2. Of these, all are mitotic poisons (on fibroblasts) at 10^{-5} M or less, except *selenite* and *diphenyhydantoin*.

So although there seems to be some sort of association between mitotic inhibition and lymphocyte poisoning, eight out of the twelve poisons tested having both actions, the association is not universal for there are two clear-cut exceptions either way. Quinone-hydroquinone, the most powerful mitotic poison known (for fibroblasts) is not toxic to lymphocytes at 10^{-4} M. Likewise chelidonine, a more powerful antimitotic than nitrogen mustard, is scarcely toxic to lymphocytes at 10^{-4} M. Contrariwise, selenite which is about as toxic to lymphocytes as nitrogen mustard and diphenylhydantoin which is almost as toxic as cortisone are not mitotic poisons.

On the other hand it must be pointed out that rotenone is both the most powerful lymphocyte poison yet recorded and also the most potent mitotic inhibitor of sea urchin eggs. And further, in the case of six poisons (rotenone, colchicine, arsenite,

nitrogen mustard, acriflavine, stilboestrol) the concentrations required for 50 per cent lymphocyte killing on the one hand and mitotic poisoning of fibroblasts on the other are practically the same.

It is remarkable that the chromosome poisons and the spindle poisons seem to be about equally good at killing lymphocytes. Now preventing the onset of mitosis would seem to be a very different proposition from destroying the spindle of a cell which has

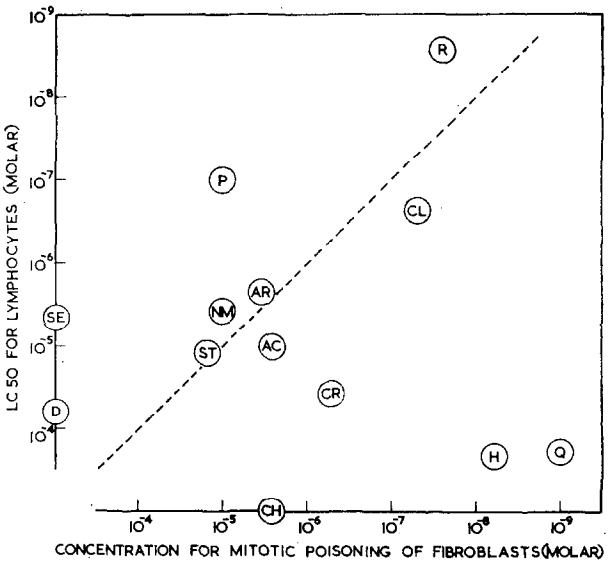

FIG. 3. Concentration which kills lymphocytes plotted against concentration which poisons mitosis of fibroblasts (both *in vitro*) for the various poisons. Lettering as for Fig. 2.

already passed through prophase, so much so that it is difficult to visualize any common biochemical lesion underlying such diverse phenomena as chromosome poisoning, spindle poisoning and lymphocyte killing. Taken as a whole, the results reported here would not support such a hypothesis. The wide chemical diversity of the effective poisons also speaks against it. If at this point we recall that ionizing radiation is also a mitotic inhibitor (acting like a chromosome poison) and a selective killer of lymphocytes, it is to point out that the task of extending the common biochemical lesion to include radiation as well is even more forbidding.

Such rough correlation as exists between mitotic poisoning and lymphocyte poisoning may be no more than a simple consequence of the fact that mitosis is the cell process and the lymphocyte the cell type most susceptible to adverse conditions.

On the general problem of the peculiar sensitivity of the lymphocyte to ionizing radiation and chemical poisons, the search for common chemical denominators seeming unfruitful, attention must be directed more to intrinsic features of the lymphocyte itself, particularly its metabolism. In this connexion in 1955 the writer[50] suggested that the relative paucity of mitochondria might be a significant point and subsequent measurements[51] showed the total mitochondrial volume of a lymphocyte to be 0·5–1·3 μ^3, compared with 800 μ^3 for a liver cell. Following this Creasey and Stocken[52]

showed that the nuclei of lymphocytes (and possibly some other radiosensitive cells) differed from those of other cells in that they had a phosphorylating system and could synthesize ATP, so they were normally not dependent on mitochondrial ATP. They also showed that this nuclear phosphorylating system was extremely sensitive to X-radiation. It is to specific differences in the intracellular localization of enzyme systems such as these that the sensitivity of the lymphocyte to radiation and mitotic poisons must ultimately be related.

It now remains to comment on some of the individual lymphocyte poisons. The three most powerful, rotenone, podophyllotoxin and colchicine are complex aromatic substances derived from plants. Rotenone is the active principle of the root of *Derris sp.* Originally used as a fish poison it is now widely used as an insecticide (derris dust). It is virtually harmless by mouth to warm-blooded animals,[5] probably because it is destroyed during digestion, but it can produce unpleasant symptoms when inhaled as a dust. Injected, it has about the same toxicity as cortisone (Table 1). Rotenone is the most powerful mitotic inhibitor known for sea urchin eggs (5×10^{-9} M) and it is here shown to be easily the most potent lymphocyte poison so far described. The toxicity of rotenone for lymphocytes is truly remarkable, it kills 20 per cent of cells at a concentration of 10^{-9} M, which, assuming a uniform distribution throughout cells and medium, amounts to only 100 molecules per cell. Prior to this discovery the lowest cytocidal concentration recorded for any chemical on any mammalian cell was 10^{-6} M for cortisone on lymphocytes.[8] Podophyllotoxin and colchicine are thirty times more active than this, while rotenone is a thousand times more active.

Leblond and Segal[54] reported that injection of colchicine produced great lymphoid atrophy in normal rats, but no effect in adrenalectomized ones. They therefore concluded that the lympholytic action of colchicine was entirely mediated by the adrenal cortex and that injection of colchicine was one of the most "alarming" stimuli known. The results obtained here show a direct effect of colchicine on lymphoid tissue at very low concentrations (10^{-7} M) and are difficult to reconcile with the findings of Lebond and Segal. The dose-effect curve for colchicine has a rather peculiar shape (Fig. 2) which seems to imply a hard core of lymphocytes which are relatively resistant to the drug. Murray *et al.*[55] showed that the metaphase arrest produced by colchicine in fibroblast cultures could be prevented or reversed by *m*-inositol. In our experiments, however, we found that the lymphocytocidal effect of 10^{-6} M colchicine was not prevented at all by 10^{-4} M *m*-inositol. It should perhaps be mentioned that the lymphocyte pyknosis produced in these experiments by colchicine and other spindle poisons was ordinary pyknosis and did not resemble the "clumped metaphases" produced in dividing tissues.

The figures for nitrogen mustard are in good agreement with those of Schrek who, working with lymphocyte suspensions *in vitro*, found the minimum concentration required to kill some cells to be 3×10^{-6} M for rabbit lymphocytes[56] and 6×10^{-6} M for human lymphocytes.[57]

The results obtained with quinone and hydroquinone were essentially identical, as has also been reported for the antimitotic action on fibroblasts[58] and sea urchin eggs[32]. This indicates that the effect on the cells is not produced by oxidation in the one case or reduction in the other. It also shows that the low toxicity found in these experiments cannot be accounted for by oxidation or reduction of the compound before it reached the cells.

The biochemical basis of selenite poisoning is still unknown. Although it has been shown to inhibit some SH enzymes *in vitro*[59] this cannot account for all its properties. SH reactants in general inhibit mitosis, but selenite does not. Selenite, arsenite and nitrogen mustard were about equally toxic to lymphocytes. Acriflavine and stilboestrol were almost identical and had about one third the toxicity of nitrogen mustard.

Diphenylhydantoin is a central nervous depressant used in the treatment of epilepsy. Clinical reports suggested that this drug may cause lymphopenia[60] and that is why it was investigated here. It was of further interest because we had previously shown[9] that barbiturates are selectively toxic to lymphocytes—but only in the range 10^{-4}–10^{-3} M which is rather higher than the concentration range considered here.

Acknowledgements—I am greatly indebted to Dr. Ivor Cornman of Hazleton Laboratories Inc., Falls Church, Va., U.S.A. for suggesting the trial of rotenone and for much information on the mitotic inhibition of sea eggs. I am grateful to Prof. J. S. Mitchell for drawing my attention to the lymphopenic action of diphenylhydantoin.

The culture work was carried out by Mr. W. R. Lush and Miss E. Peakman, and the cell counting by Mr. W. R. Lush. Miss M. Tebbutt made the film preparations and gave general assistance. It is a pleasure to thank these technicians for their very careful and painstaking work. I have to thank Messrs. Parke, Davis & Co. for a gift of pure sodium-5 : 5-diphenylhydantoin ("epanutin").

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