THE EFFECT OF PODOPHYLLOTOXIN ON TISSUE METABOLISM AND ENZYME SYSTEMS*

By ZELMA BAKER MILLER, Ph.D., CLARKE DAVISON, and PAUL K. SMITH, Ph.D.

(From the Department of Pharmacology, The George Washington University School of Medicine, Washington, D. C.)

(Received for publication, July 27, 1949)

INTRODUCTION

The crude drug, podophyllin, which is derived from the May-apple root, has been used for many years as a cathartic agent. The carcinoclastic action and effectiveness of this material against condylomata acuminata (venereal warts) have only recently been described. Kaplan (1) and subsequent workers (2–7) produced involution of condylomata acuminata by the local application of a suspension of podophyllin in oil. Culp and Kaplan (3) obtained dramatic results in two hundred cases within 2 to 3 days after a single application of podophyllin, and noted little or no effect on surrounding normal tissue. Reich and coworkers (8) reported necrosis and sloughing of soft papillomas of the female urethra after local application of podophyllotoxin, one of the pure constituents of podophyllin, and Tomskey, Vickery, and Getzoff successfully treated granuloma inguinale in twenty patients (9). Ormsbee, Cornman, and Berger (10) observed selective damage of tumor cells in tissue culture at podophyllin concentrations of 0.08 to 20 mg. per liter, and Belkin (11) found that subcutaneous injection of podophyllin into the mouse caused a marked retardation in the rate of growth of a transplanted tumor (sarcoma 180). The chick embryo is so sensitive to podophyllotoxin that 1 gamma, injected into the yolk sac of the egg, is fatal (12).

The changes in the mitotic activity and patterns of squamous epithelia induced by podophyllin (6) suggest a direct and specific effect on the dividing cell. This conclusion is strengthened by the observations that podophyllin blocks mitosis in the root tips of *Allium cepa* (13), and destroys the mitotic spindles in cleaving *Asterias* and *Arbacia* eggs (14).

Despite the unusual biological properties and the carcinoclastic action of podophyllotoxin (15), the effects of this interesting compound on enzymes and tissue metabolism have received little attention.

Waravdekar and Leiter (16) have reported recently that 10 to 100 mg. of podophyllotoxin per liter *in vitro* did not significantly affect the respiration of tissue homogenates, but that the subcutaneous injection of 20 mg. per kg.

*We wish to acknowledge the assistance of a grant from the National Cancer Institute of the United States Public Health Service.

into tumor-bearing mice caused a marked inhibition of the cytochrome oxidase activity of sarcoma 37. No other tissues were affected.

In the hope of determining the mechanism of action of podophyllotoxin, we have investigated the effects of this compound on various enzymes and metabolic systems. In a preliminary report of this work it was shown that 10^{-3}M podophyllotoxin inhibits the respiration of various rat tissues, and the oxidation of acetate and butyrate by rabbit kidney homogenate (17). Anaerobic glycolysis of chick embryo and rat testis was inhibited by high concentrations of podophyllotoxin, and stimulated by lower concentrations. We have extended these observations in the present study, and have also investigated other enzymatic systems which might possess important functions in growing tissue. In addition, because podophyllotoxin inhibits mitosis in *Allium cepa* (14) and acts similarly to colchicine (5), which inhibits seed germination, we have studied the effect of podophyllotoxin on the germination of radish, cucumber, and corn seeds.

Methods

Measurements of respiration and glycolysis were carried out in Warburg vessels at 37.5°C For aerobic experiments, tissue slices were immersed in Ringer-Krebs phosphate buffer, pH 7.4, and were gassed with oxygen. For anaerobic experiments, Ringer-Krebs bicarbonate buffer and a gas mixture, consisting of 95 per cent nitrogen and 5 per cent carbon dioxide, were used.

Tissue homogenates were made in phosphate-saline solution (0.02 M phosphate buffer, pH 7.4), and oxygen uptake was measured in air. Rabbit kidney homogenate, for acetate and butyrate oxidation, was prepared according to Kalnitsky and Barron (18).

All manometric experiments were performed in duplicate unless otherwise noted. Most experiments were continued for several hours because the inhibitory effect frequently appeared slowly.

The magnitude of the respiration is expressed as the Q_{O_2} value, mm.^{3} oxygen consumed per mg. dry weight of tissue per hour, and glycolysis as Q_{CO_2}, mm.^{3} CO_2 evolved per mg. dry weight per hour.

Twenty-four mg. of podophyllotoxin[1] was dissolved in 2 cc. of ethylene glycol. One-tenth cc., diluted with buffer to 3 cc., provided a final concentration of 10^{-3}M. In control studies, an equivalent amount of ethylene glycol had little or no effect. When prepared in this manner, podophyllotoxin was soluble in both phosphate and bicarbonate buffers even at a concentration of 10^{-3}M, the highest studied.

Podophyllotoxin was placed in the main vessel, and the substrate in the side arm. Substrates were added at zero time, after equilibration of the vessels in the water bath for the required period of time.

The respiration of tissues from animals injected with podophyllotoxin was also determined. Mice bearing 6 day old transplanted tumors, sarcoma 37,[2] were injected subcutaneously in the axilla opposite the tumor with podophyllotoxin, 20 mg. per kg., in 50 per cent propylene

[1]Kindly furnished by Dr. W. G. Bywater of the S. B. Penick Co., New York.

[2]We are indebted to Dr. Joseph Leiter of the National Cancer Institute for making the mice available.

glycol. They were sacrificed at various time intervals after injection. The tissues were immediately removed and sliced, and respiration was measured in the absence of added substrate. Large, normal rats (265 to 375 gm.) and weanlings, (35 to 60 gm.), were injected subcutaneously with 15 mg. per kg. of podophyllotoxin in 50 per cent propylene glycol. The respiration of spleen and lymph nodes from the large rats and of thymus glands from the weanlings was determined.

Podophyllotoxin,[3] 0.8 microgram in a volume of 0.5 cc., was injected into the yolk sacs of fertile chicken eggs, bearing 5 day old embryos.[4] The compound was dissolved in the minimum amount of alcohol and diluted with sterile saline. The eggs were opened at various intervals after injection, and the respiration of the embryo was measured.

The effect of podophyllotoxin on the germination of corn, cucumber, and radish seeds was determined as follows: 40 or 50 seeds of each variety were placed in a Petri dish on a sheet of filter paper, moistened with 5 or 10 cc. of the podophyllotoxin or control solution. The podophyllotoxin was dissolved in ethyl alcohol or ethylene glycol and diluted to the desired final concentration. All experiments were performed in duplicate. The number of sprouts which had broken through the seed coats were counted after suitable intervals of time.

RESULTS

In Vitro Experiments.—Podophyllotoxin, 10^{-3} M, inhibits the respiration of all the tissues studied. The results obtained without added substrate are indicated in Table I. In general, the inhibition increases with time. Thus, in the 1st hour, spleen is inhibited 36 per cent, in the 2nd hour, 75 per cent, and in the 3rd hour, 76 per cent. Similarly, kidney is inhibited only 2 per cent in the 1st hour, 27 per cent in the 2nd hour, and 74 per cent in the 3rd hour. In the absence of added substrate, lymph nodes, spleen, and kidney are most sensitive. Thymus and mouse sarcoma 37 are almost as sensitive. The inhibition with liver, testis, and chick embryo does not exceed 28 per cent. Brain is quite sensitive in the absence of glucose; however, the control respiration falls off so rapidly that it is difficult to evaluate this finding.

When M/100 glucose is added (Table II), the control respiration of most of these tissues is stabilized, and does not decrease so rapidly over the 3 hour period. Nevertheless, a similar inhibition, increasing progressively with time, is observed with spleen and kidney. Thus spleen is inhibited 6 per cent in the 1st hour, 32 per cent in the 2nd hour, and 59 per cent in the 3rd hour. Kidney is inhibited 16 per cent in the 1st hour, 30 per cent in the 2nd hour, and 41 per cent in the 3rd hour. Rat brain, liver, testis, and chick embryo are all inhibited in the first hour, 18, 29, 19, and 15 per cent respectively, but these values do not change significantly with time. Therefore, in the presence of glucose, spleen and kidney are considerably more sensitive than the other tissues studied.

The effect of time of exposure and concentration of podophyllotoxin on the

[3]Since 1 gamma of podophyllotoxin is fatal to the chick embryo (12) this is approximately the maximum tolerated dose.

[4]We wish to thank Dr. Mary Lou Robbins for her assistance in this procedure.

TABLE I

Effect of 10^{-3}M Podophyllotoxin on Tissue Slice Respiration

No added substrate

Temperature, 37.5°C. Oxygen atmosphere.

Tissue	Experiment	1st hr.		2nd hr.		3rd hr.	
		Q_{O_2}*	per cent inhibition	Q_{O_2}	per cent inhibition	Q_{O_4}	per cent inhibition
Rat lymph nodes	Control	6.6		6.0		4.0	
	Podophyllotoxin	2.5	62.2	2.0	66.6	0.8	80.1
Rat spleen	Control	16.0		10.0		6.6	
	Podophyllotoxin	10.2	36.2	2.5	75.0	1.6	75.8
Rat kidney	Control	19.1		16.3		12.5	
	Podophyllotoxin	18.7	2.1	11.8	27.6	3.2	74.2
Rat thymus	Control	11.2		9.9		7.1	
	Podophyllotoxin	12.2	+8.9	6.8	31.4	3.4	52.2
Rat brain	Control	6.5		2.5		—	
	Podophyllotoxin	2.0	69.0	1.3	48.0	—	
Rat liver	Control	14.9		13.1		9.7	
	Podophyllotoxin	13.1	12.1	9.7	26.0	7.0	28.0
Rat testis	Control	7.8		3.5		1.8	
	Podophyllotoxin	6.7	14.1	2.7	22.8	1.7	6.7
Rabbit lymph nodes	Control	5.5		5.3		4.7	
	Podophyllotoxin	3.0	45.0	2.4	55.0	1.8	62.0
Chick embryo (5 day)	Control	13.2		10.5		9.3	
	Podophyllotoxin	9.9	25.0	7.7	26.2	8.8	5.4
Mouse tumor (sarcoma 37)	Control	10.3		9.2		7.5	
	Podophyllotoxin	8.2	20.4	4.0	56.5	2.9	61.3

Podophyllotoxin dissolved in ethylene glycol. Control vessels contain corresponding amount of ethylene glycol.

All values represent average of duplicates.

* Q_{O_2} = mm.^{3} oxygen consumed per mg. dry weight of tissue per hour.

respiration of rat spleen slices is shown in Fig. 1. The respiration of spleen is inhibited within the first 30 minutes by 10^{-3}M podophyllotoxin and the inhibition increases steadily with time. Both 10^{-4} and 10^{-5}M initially stimulate spleen respiration slightly; after $2\frac{1}{2}$ or 3 hours' exposure, these concentrations inhibit.

TABLE II

Effect of 10^{-3} M Podophyllotoxin on Tissue Slice Respiration

M/100 glucose

Temperature, 37.5°C. Oxygen atmosphere.

Tissue	Experiment	1st hr.		2nd hr.		3rd hr.	
		Q_{O_2}*	per cent inhibition	Q_{O_2}	per cent inhibition	Q_{O_2}	per cent inhibition
Rat spleen	Control	12.2		12.7		11.1	
	Podophyllotoxin	11.4	6.5	8.6	32.3	4.5	59.4
Rat kidney	Control	25.6		24.3		21.6	
	Podophyllotoxin	21.4	16.4	17.1	29.7	12.7	41.3
Rat brain	Control	14.7		15.8		15.7	
	Podophyllotoxin	12.0	18.4	12.5	20.9	13.3	15.3
Rat liver	Control	17.5		15.3		10.7	
	Podophyllotoxin	12.4	29.1	10.7	30.2	7.4	30.9
Rat testis	Control	7.9		8.1		—	
	Podophyllotoxin	6.4	19.0	5.9	27.2	—	
Chick embryo	Control	13.0		10.6		11.2	
	Podophyllotoxin	11.0	15.4	9.9	6.6	9.7	13.4

* Q_{O_2} = mm.^{3} oxygen consumed per mg. dry weight of tissue per hour.

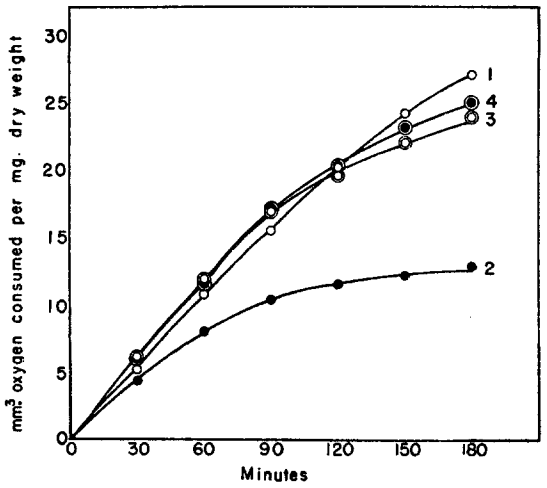

FIG. 1. Effect of podophyllotoxin on the respiration of rat spleen. (1) control; (2) 10^{-3}M podophyllotoxin; (3) 10^{-4}M podophyllotoxin; (4) 10^{-5}M podophyllotoxin.

The effect of podophyllotoxin on the oxidation of various substrates has been studied with kidney slices. This tissue was chosen because it can oxidize many intermediary metabolites. The Q_{O_2} values and the per cent inhibition

TABLE III

Rat Kidney Slices

Effect of 10^{-3} M Podophyllotoxin on Oxidation of Various Substrates

Temperature, 37.5°C. Oxygen atmosphere.

Substrate	Conditions	1st hr.		2nd hr.		3rd hr.	
		Q_{O_2}	per cent inhibition	Q_{O_2}	per cent inhibition	Q_{O_2}	per cent inhibition
None	Control	25.2		18.9		12.5	—
	Podophyllotoxin	18.7	27.0	13.4	28.1	7.5	40.0
Acetate 0.02 M	Control	26.7		21.0		14.4	—
	Podophyllotoxin	29.9	+12.0	18.8	10.5	8.7	39.6
None	Control	27.1		20.1		14.8	—
	Podophyllotoxin	19.8	26.9	14.6	27.4	9.7	34.4
Glucose 0.01 M	Control	25.6		24.3		21.6	—
	Podophyllotoxin	21.4	16.4	17.1	28.1	12.7	41.0
Alanine 0.01 M	Control	40.1		37.5		34.3	—
	Podophyllotoxin	29.3	26.9	26.0	30.8	21.9	36.0
None	Control	23.6		18.3		15.3	—
	Podophyllotoxin	18.0	23.8	6.9	62.2	2.6	83.0
Glutamate 0.01 M	Control	27.8		26.9		24.1	—
	Podophyllotoxin	28.9	+3.9	21.9	19.2	8.9	63.0
Succinate 0.01 M	Control	44.7		43.2		38.2	—
	Podophyllotoxin	50.2	+12.3	41.1	4.9	29.1	24.8
None	Control	27.4		20.9		14.7	—
	Podophyllotoxin	18.5	32.5	11.1	47.0	8.3	43.5
Pyruvate 0.01 M	Control	36.6		38.0		33.7	—
	Podophyllotoxin	41.5	+13.4	43.4	+14.2	27.0	19.8

for the 1st, 2nd, and 3rd hours are indicated in Table III. In the presence of acetate, succinate, and pyruvate, 10^{-3}M podophyllotoxin stimulates in the 1st hour, but inhibits in the 2nd and 3rd hours. The inhibition of acetate, glucose, alanine, and glutamate oxidation approximates that observed in the control

during the 3rd hour. However, with succinate and pyruvate, the degree of inhibition, even in the 3rd hour, is considerably less than that of the control slices (25 per cent for succinate and 83 per cent for the control; 20 per cent for pyruvate and 44 per cent for the control).

Since rabbit kidney homogenate offered a more convenient preparation than rat kidney slices, the oxidation of acetate, butyrate, and glucose by this preparation was investigated. Rabbit kidney cortex was ground in the cold with

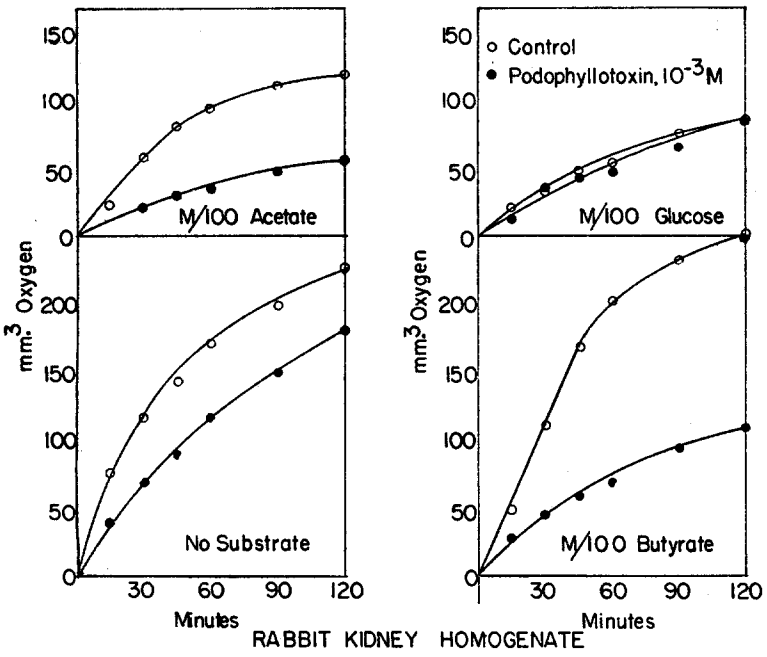

FIG. 2. Effect of podophyllotoxin on oxygen consumption by rabbit kidney homogenate. O = control; ● = 10^{-3}M podophyllotoxin.

cold phosphate-saline buffer, 4 gm. tissue per 40 cc. solution (18). Two cc., equivalent to 200 mg. of tissue, was pipetted into each vessel. Substrates were added from the side-arm. Some typical results are shown in Fig. 2. The values in the acetate, butyrate, and glucose curves have been corrected for the control values without added substrate, and therefore represent the increment in oxygen consumption due to these substances. Glucose oxidation by rabbit kidney homogenate is not inhibited by 10^{-3}M podophyllotoxin. On the other hand, both acetate and butyrate oxidations are quite sensitive: acetate oxidation is inhibited 43 per cent in the 1st hour, and 40 per cent in the 2nd hour; butyrate oxidation is inhibited 50 per cent in the 1st hour and 60 per cent in the 2nd hour.

Fig. 3 and Table IV show the effect of podophyllotoxin on the anaerobic glycolysis of chick embryo, rat brain, and rat testis in the presence of M/100 glucose. Podophyllotoxin, 10^{-3}M, inhibits chick embryo glycolysis 59 per cent in the 1st hour and 56 per cent in the 2nd hour; 10^{-4}M podophyllotoxin inhibits 15 and 11 per cent in the 1st and 2nd hours; 10^{-5} and 10^{-6}M podophyllotoxin stimulate about 20 per cent. Rat brain glycolysis is inhibited by 10^{-3}M podophyllotoxin, but the inhibition is not so marked as with embryo, amounting to only 18 per cent. However, considerable stimulation is observed with the lower concentrations; the maximum, 40 per cent, is produced by 10^{-5}M podophyllotoxin. Glycolysis by rat testis is inhibited nearly 40 per cent by 10^{-3}M

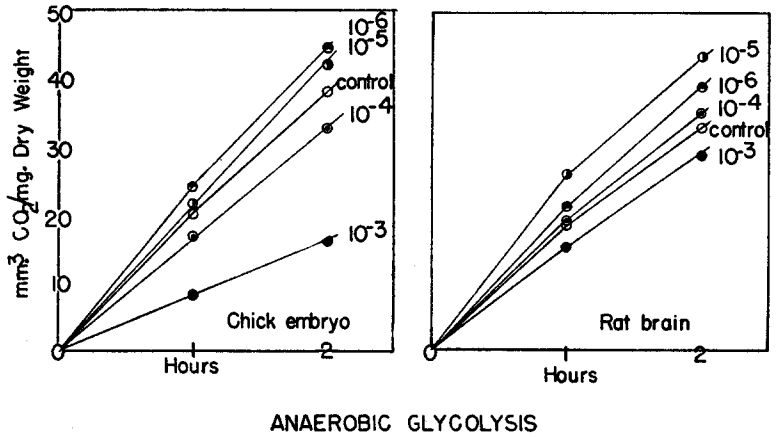

FIG. 3. Effect of podophyllotoxin on anaerobic glycolysis of chick embryo and rat brain. ○ = control; ● = 10^{-3}M; ◉ = 10^{-4}M; ◑ = 10^{-5}M; ◓ = 10^{-6}M.

podophyllotoxin; the maximum stimulation, 14 per cent, is observed with 10^{-6}M podophyllotoxin.

Podophyllotoxin, 10^{-3}M, has no effect on a number of other enzyme systems studied. These include five oxidases, (succinoxidase, choline, xanthine, tyrosine, and leucine oxidases), alkaline and acid phosphatase, adenosine triphosphatase, and choline esterase (Table V). Podophyllotoxin, 10^{-3}M, did not inhibit the formation of acid-soluble phosphorus from either ribonucleic acid or thymonucleic acid, nor the reduction in viscosity which precedes the splitting off of phosphorus. Succinoxidase was selected as a typical representative of the sulfhydryl enzymes (19, 20) and choline oxidase because Barron, Bartlett, and Miller (21) had found it was extremely sensitive to nitrogen mustards, and concluded that it might be involved in the antimitotic action of these compounds. Xanthine oxidase and the nucleic acid depolymerases were studied because of the importance of nucleic acids and their building stones in cell

TABLE IV

Effect of Podophyllotoxin on Anaerobic Glycolysis

M/100 glucose

Temperature, 37.5°C. 95 per cent N_2, 5 per cent CO_2.

Tissue	Experiment	1st hr.		2nd hr.	
		Q_{CO_2}*	per cent inhibition	Q_{CO_2}	per cent inhibition
Chick embryo (5 days)	Control	20.2	—	18.2	—
	10^{-3}M podophyllotoxin	8.3	59.0	8.0	55.9
	10^{-4}M "	17.1	15.4	16.2	−11.0
	10^{-5}M "	21.8	+7.9	20.7	+13.7
	10^{-6}M "	24.2	+19.8	20.9	+14.8
Rat brain	Control	18.6	—	14.2	
	10^{-3}M podophyllotoxin	15.3	−17.8	13.4	5.6
	10^{-4}M "	18.9	+1.6	16.4	+15.5
	10^{-5}M "	26.0	+39.8	17.5	+23.2
	10^{-6}M "	21.3	+14.5	17.9	+26.1
Rat testis	Control	6.3	—	5.8	—
	10^{-3}M podophyllotoxin	3.8	−39.6	3.5	39.6
	10^{-4}M "	5.4	−14.0	4.2	27.6
	10^{-5}M "	6.6	+5.0	5.4	6.9
	10^{-6}M "	7.2	+14.0	6.0	+3.0

* Q_{CO_2} = mm.³ carbon dioxide evolved per mg. dry weight per hour.

TABLE V

Enzymes Unaffected by 10^{-3} M Podophyllotoxin

Enzyme	Source	Substrate	Method of determination
Succinoxidase	Pigeon breast muscle	Succinate	Oxygen consumption
Choline oxidase	Rat liver homogenate	Choline	" "
Xanthine oxidase	" " "	Xanthine	" "
Tyrosine oxidase	" " "	Tyrosine	" "
Leucine oxidase	*Proteus vulgaris*	Leucine	" "
Alkaline phosphatase	Dog serum	β-Glycerophosphate	Inorganic P formation
Acid phosphatase	" "	"	Inorganic P formation
Adenosine triphosphatase	Rat liver	Adenosine triphosphate	Inorganic P formation
Choline esterase	Rat brain homogenate	Acetyl choline	Acid liberation
Ribonucleodepolymerase	Spleen mince	Ribonucleic acid	Acid-soluble phosphorus
Thymonucleo-depolymerase	" "	Thymonucleic acid	" "
"	Dog serum	" "	Viscosity change

multiplication, and the phosphatases because of their rôle in phosphate transfer and synthesis of cellular structural elements.

It has been reported that both x-rays and nitrogen mustards reduce the viscosity of thymonucleic acid in the absence of a specific enzyme (22, 23). It was of interest, therefore, to determine whether or not podophyllotoxin, which also inhibits mitosis of cells, possesses the same property. It was found that concentrations of podophyllotoxin as high as 3×10^{-3}M had no effect on the viscosity of thymonucleic acid.

TABLE VI

Effect of Podophyllotoxin Injection on the Respiration of Tissues from Tumor-Bearing Mice

Normal					Time after injection	Podophyllotoxin-treated			
Q_{O_2}						Q_{O_2}			
Mice	Liver	Kidney	Spleen	Tumor		Liver	Kidney	Spleen	Tumor
					hrs.				
1	8.2	23.5	21.0	9.4	2	6.1	22.2	20.3	3.7
2	8.8	23.0	19.3	7.7	2	9.0	23.8	13.7	3.5
3	5.0	23.6	18.4	8.3	4	4.6	26.9	11.7	1.8
4	6.6	20.5	17.3	11.3	4	6.7	23.4	13.1	0.9
5	7.5	19.0	18.1	6.7	6	9.6	21.6	9.2	0.1
6	10.3	21.5	18.6	11.9	6	11.3	21.2	7.6	0.3
7	9.9	—	17.4	12.2	8	6.2	23.6	10.6	0.4
8	7.3	23.3	24.1	14.4	8	11.9	23.2	10.3	0.6
9					24	6.9	21.6	8.0	
10					24	9.9	23.0	9.1	
Average.....	8.0	22.0	19.3	10.2					

The mice were injected subcutaneously with 20 mg. per kg. of podophyllotoxin in 50 per cent propylene glycol.

In Vivo Experiments.—In an effort to confirm the finding of Waravdekar and Leiter (16) that podophyllotoxin injection into tumor-bearing mice produces a specific inhibition of the respiration of tumor homogenate, the following experiment was performed: 10 mice which had been inoculated 6 days earlier with sarcoma 37 were injected subcutaneously with 20 mg. per kg. of podophyllotoxin in 50 per cent propylene glycol. This is approximately the maximum tolerated dose. The mice were killed after 2, 4, 6, 8, and 24 hours, and the respiration of liver, kidney, spleen, and tumor slices was determined, without added substrate. Eight uninjected mice served as controls.

The tumors were somewhat hemorrhagic after injection and the amount of hemorrhage increased with time. In some animals, the spleen decreased in size. Spleen weight/total weight ratios were not determined in these experi-

ments. Care was taken to select the least necrotic slices of tumor tissue for the Warburg experiments. The results obtained are shown in Table VI and in Fig. 4. The liver and kidney values from treated mice did not differ significantly from those from untreated mice: liver values ranged from 4.6 to 11.9 compared with an average of 8.0 (range 5.0 + 10.3) in the control animals; kidney values ranged from 21.2 to 26.9, compared with an average of 22.0 (range 19.0 + 23.6) in control animals. There was no indication of any consistent change in relation to time after injection. However, striking diminution of tumor and spleen respiration was observed: 2 hours after injection, the Q_{O_2} for tumors in two mice was 3.7 and 3.5 (untreated, 10.2); 4 hours after injection, the values had decreased to 1.8 and 0.9. Values obtained after that

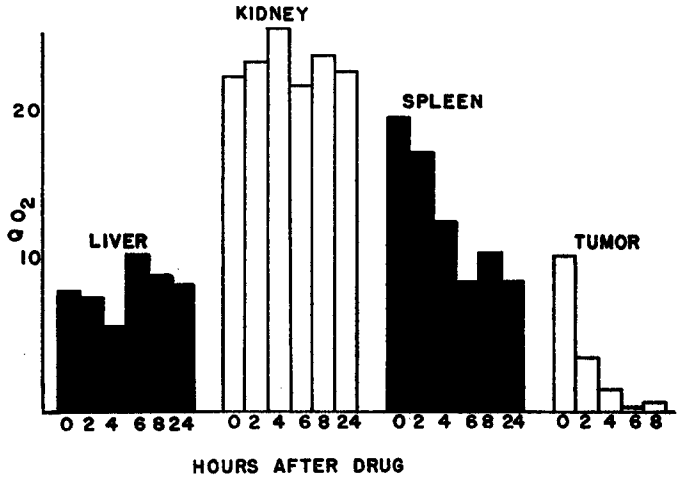

FIG. 4. Effect of podophyllotoxin on tissue respiration of tumor-bearing mice.

time ranged from 0.1 to 0.6, compared with the normal average of 10.2. Similarly, the Q_{O_2} for spleen for two mice 2 hours after injection was 20.3 and 13.7 (untreated, 21.0 and 19.3); all subsequent values were distinctly lower than the lowest normal value observed, 17.3, and ranged from 7.6 to 13.7. It is apparent, therefore, that both tumor and spleen are sensitive to podophyllotoxin *in vivo*. The results with tumor confirm those of Waravdekar and Leiter (16).

In order to extend these observations, and to determine whether other lymphatic tissues are similarly inhibited, the effect of podophyllotoxin injection on the respiration of rat spleen, lymph nodes, and thymus glands was investigated. Large, mature rats (265 to 375 gm.) and immature rats, weighing 35 to 60 gm., were injected subcutaneously with podophyllotoxin, 15 mg. per kg. in 50 per cent propylene glycol. At various time intervals after injection spleen

and lymph nodes were removed from the large rats, and thymus, and in some cases, spleen, from the small rats. Spleen and lymph nodes had usually decreased in size, but the thymus appeared normal. The rate of respiration of slices of these tissues was determined (Table VII). It was found that spleen

TABLE VII

Effect of Podophyllotoxin Injection on the Respiration of Spleen, Lymph Nodes, and Thymus Glands from Normal Rats

Time after injection	Spleen		Lymph nodes		Thymus gland*	
	No. of rats	Q_{O_2}	No. of rats	Q_{O_2}	No. of rats	Q_{O_2}
hrs.						
0	14	11.6 (10.1–12.8)	6	8.3 (5.2–9.6)	8	12.9 (10.4–17.3)
6	6	8.3 (8.0–9.2)	5	5.1 (4.2–6.2)	5	8.8 (5.7–11.1)
24	11	6.5 (2.6–9.2)	6	4.5 (2.8–5.8)	6	4.4 (2.8–5.4)

20 mg. per kg. of podophyllotoxin in 50 per cent propylene glycol injected subcutaneously.
* Thymus glands obtained from rats weighing 40 to 65 gm.

TABLE VIII

Effect of Podophyllotoxin Injection into Chicken Eggs on the Metabolism of 5 Day Old Chick Embryos

Time after injection	No. of embryos	Q_{O_2}	
		Average	Range
hrs.			
Controls	12	11.3	9.4–13.1
2	5	11.8	9.8–12.4
4	5	10.8	9.8–12.3
6	5	10.7	9.1–11.9
8	4	11.1	9.5–12.9
24	1	9.5	—

0.8 microgram podophyllotoxin in sterile saline injected aseptically into the yolk sac.

respiration was inhibited 28 per cent after 6 hours and 44 per cent after 24 hours; lymph nodes 38 per cent after 6 hours and 46 per cent after 24 hours and thymus, 32 per cent after 6 hours and 66 per cent after 24 hours. The injection of propylene glycol alone into control rats had no effect on the respiration of these tissues.

A similar experiment was performed with fertile chicken eggs, bearing 5 day old chick embryos. Podophyllotoxin, 0.8 microgram in 0.5 cc. sterile saline,

was injected into the yolk sac under aseptic conditions. Controls were injected with 0.5 cc. sterile saline. The eggs were opened at 2, 4, 6, 8, and 24 hour intervals after injection, and the respiration of each embryo was determined, one per vessel (Table VIII).

TABLE IX

Effect of Podophyllotoxin on Seed Germination

Seeds	Solvent	Time	No. of seeds germinated*			
			Control	0.01 per cent podophyllotoxin	Control	0.001 per cent podophyllotoxin
		hrs.				
Radish	Ethyl alcohol	17	9	11	27	29
		20	21	23	34	38
Cucumber	Ethyl alcohol	21	40	39	43	40
Corn	Ethyl alcohol	19	26	29	31	34
			Control	0.1 per cent podophyllotoxin	Control	0.2 per cent podophyllotoxin
Radish	Ethylene glycol	17	23	17	8	7
		22	33	31	14	12
Cucumber	Ethylene glycol	17	3	4	1	1
		19	14	12	2	3
Corn	Ethyl alcohol	19	28	31	—	—
		23	34	36	—	—
	Ethylene glycol	19	13	19	—	—
		23	18	20		

* Average of two separate experiments.

The average value for the respiration of 12 saline-injected embryos was 11.3. Embryos taken 2, 4, 6, and 8 hours after injection of 0.8 microgram of podophyllotoxin into the egg showed completely normal values: 11.8, 10.8, 10.7, and 11.1. After 24 hours, only one of five podophyllotoxin-treated embryos survived, but this also was essentially normal, $Q_{O_2} = 9.5$. These data are surprising in view of the fact that 0.8 microgram is the maximum tolerated dose, and 1.0 microgram is lethal.

Seed Germination.—Podophyllotoxin, 0.001, 0.01, 0.1, and 0.2 per cent, had no effect on the germination of radish or cucumber seeds or corn kernels, compared with controls exposed to similar dilutions of the solvent, alcohol or ethylene glycol (Table IX).

DISCUSSION

These experiments were undertaken in the hope that they would increase our understanding of the mechanism by which compounds such as podophyllotoxin influence mitotic processes and destroy rapidly dividing cells. This might permit the formulation of generalizations which would also apply to other carcinoclastic agents, and might provide simple, *in vitro* techniques for screening potential carcinoclastic compounds. The rapid and specific action of podophyllin and some of its constituents on certain malignant growths and condylomata acuminata, and the bizarre changes observed in mitotic patterns suggest that, in all probability, the compound acts specifically on an enzyme or metabolic system, essential for the synthesis of cellular constituents or required for the provision of energy.

Our current understanding of the cell in mitosis is too limited to permit an exact listing of all the enzymes present. Many enzymes which are found in the mature cell are also present in the rapidly dividing cell. However, their proportions and relative importance may differ considerably. The additional enzymes, if any, and the special conditions or hormone-like controlling agents which initiate the dramatic events involved in the actual division of cells are unknown. The great sensitivity of malignant and other rapidly dividing cells to compounds such as podophyllotoxin suggests the possibility that normal and malignant cells may actually differ *qualitatively* in their enzymatic composition.

Because we do not know all the enzymes and conditions which regulate cell multiplication and division, it is necessary to study enzymes which are known to function in normal adult tissues. This is in no sense a futile effort, for the study of specific inhibitors of cell division may demonstrate that certain metabolic processes which occur normally in the adult cell may assume special importance in rapidly dividing cells, due to unusual energy or other demands. Thus, in experiments with nitrogen mustards [which, like podophyllotoxin, are also carcinoclastic agents (24)], Barron, Bartlett, and Miller (21) showed that choline oxidase, an enzyme of normal, adult tissue, is almost completely inhibited by nitrogen mustards *in vitro* at concentrations far below those required for *in vivo* effects. Exposure of rats to nitrogen mustards markedly or completely inhibited several enzymes involved in synthetic reactions as well as pyruvic acid oxidation *in vitro* (20). From these data, the conclusion was drawn that these metabolic systems may play an important rôle in cell multiplication, a fact which may explain the sensitivity to nitrogen mustards.

In the experiments reported here, the action of podophyllotoxin was investigated on several oxidases, including succinoxidase, some phosphatases, including adenosine triphosphatase, and certain hydrolytic enzymes, including several involved in nucleic acid metabolism. These were chosen in this initial

orienting study because they are representative of certain classes of enzymes, vital to cell function and probably to cell multiplication. None of the series of enzymes studied was inhibited. Assuming that these or similar enzymes function in the dividing cell, our experiments probably rule out the possibility that podophyllotoxin effects its action by inhibiting succinoxidase, choline oxidase, adenosine triphosphatase, the depolymerases for ribo- or desoxyribonucleic acids, or any of the others investigated.

On the other hand, the glycolysis and respiration of tissue slices, either from animals treated with podophyllotoxin or in the presence of added podophyllotoxin, were very definitely affected. The anaerobic glycolysis of chick embryo was inhibited by 10^{-3}M podophyllotoxin; it was stimulated at higher concentrations. Similar results were obtained with brain and testis glycolysis. Podophyllotoxin *in vitro* inhibited the respiration of rat lymph nodes, spleen, thymus, and kidney, and mouse tumor to a considerably greater extent than that of rat liver, brain, testis, and chick embryo. The respiration of lymph nodes was most sensitive. Moreover, after injection of podophyllotoxin into both mice and rats, the respiration of tumor, spleen, lymph nodes, and thymus gland was markedly reduced. Some effect was demonstrable 2 hours after injection of the compound. It is apparent from our results that the respiration of lymphatic tissue is unusually sensitive to podophyllotoxin.

The marked sensitivity of lymphatic tissue and the increase in the degree of inhibition with time recall results obtained with nitrogen mustards (25). Perhaps a more detailed study of the metabolism of lymphatic tissue and its response to various drugs will provide new information about tumor metabolism and growth, expecially tumors of the hematopoietic system. Both types of tissue are characterized by potentialities for rapid changes in size and the synthesis of large amounts of nucleic acids and other cellular constituents.

Involution of lymphatic tissue has been observed after treatment with nitrogen mustards (26). Similarly, Kelly, MacCardle, and Smith (27) noted involution of the mouse spleen and other phenomena suggestive of Selye's alarm reaction (28) after the subcutaneous injection of podophyllotoxin. In 1933 Dustin (29) described the so called crisis of nuclear fragmentation, characterized by a sudden increase in the number of pyknoses, especially in the thymus and lymph glands of animals treated with specific drugs. Selye, however, has suggested that the crisis of nuclear fragmentation is merely another manifestation of the alarm reaction, due to involution of lymphatic tissue and caused by excess corticoids (28). Experiments to be reported (30) indicate that injection of colchicine and one of the nitrogen mustards into rats similarly reduces the respiration of lymphatic tissue. It is conceivable that such inhibition is a very early manifestation of the alarm reaction, apparent even before gross changes in size or pathology are discernible.

SUMMARY

Podophyllotoxin, 10^{-3}M, inhibits the respiration *in vitro* of rat lymph nodes, thymus, kidney, tumor, spleen, liver, brain, testis, and chicken embryo. Lymph node and spleen respiration are most sensitive, and the degree of inhibition increases with time.

The injection of podophyllotoxin into tumor-bearing mice (20 mg. per kg.) causes a dramatic reduction in the respiration of tumor slices. Within 6 hours, the respiration approaches zero. Inhibition is evident 2 hours after injection of the drug. Spleen respiration is reduced 50 per cent within 6 hours. Kidney and liver respirations remain within normal limits.

Marked reductions in the respiration of spleen, lymph nodes, and thymus glands of normal rats are produced by the injection of 15 mg. per kg. Thymus gland is the most sensitive of these three tissues, and its respiration is reduced 66 per cent 24 hours after injection of the drug.

The injection of 0.8 microgram podophyllotoxin into the yolk sac of chicken eggs bearing 5 day embryos has no effect on the respiration of the embryo within 8 hours, although this is a sufficiently toxic dose to kill 80 per cent of the embryos (within 24 hours).

Kidney respiration in the presence of acetate, glucose, alanine, and glutamate is inhibited to approximately the same degree as in the absence of added substrate. Succinate and pyruvate oxidation by rat kidney slices appear to be less sensitive.

Oxidation of acetate and butyrate by rabbit kidney homogenate is more sensitive to podophyllotoxin than oxidation by rabbit kidney homogenate without added substrate. Glucose oxidation by this preparation is not inhibited by 10^{-3}M podophyllotoxin.

The anaerobic glycolysis of chicken embryo, rat brain, and rat testis is stimulated by 10^{-5} and 10^{-6}M podophyllotoxin, and is inhibited by 10^{-3}M.

The following enzymes are not inhibited by 10^{-3}M podophyllotoxin: succinoxidase from pigeon breast muscle, choline, xanthine and tyrosine oxidase from rat liver homogenate, and leucine oxidase from *Proteus vulgaris;* alkaline and acid phosphatase from dog serum; adenosine triphosphatase from rat liver; choline esterase from rat brain homogenate; ribonucleodepolymerase from spleen mince and thymonucleodepolymerase from dog serum.

High concentrations of podophyllotoxin do not influence the viscosity and degree of polymerization of thymonucleic acid.

BIBLIOGRAPHY

1. Kaplan, I. W., *New Orleans Med. Surg. J.*, 1942, **94,** 388.
2. Culp, O. S., Magid, M. A., and Kaplan, I. W., *J. Urol.*, 1944, **51,** 655.
3. Culp, O. S., and Kaplan, I. W., *Ann. Surg.*, 1944, **120,** 251.
4. MacGregor, J. V., *Brit. Med. J.*, 1945, **1,** 593.

5. King, L. S., and Sullivan, M., *Science*, 1946, **104,** 244.
6. King. L. S., and Sullivan, M., *Arch. Path.*, 1947, **43,** 374.
7. Sullivan, M., and Blanchard, K., *Bull. Johns Hopkins Hosp.*, 1947, **81,** 65.
8. Reich, W. J., Nechtow, M. J., and Rubenstein, M. W., *Am. J. Obst. and Gynec.*, 1947, **53,** 658.
9. Tomskey, G. C., Vickery, G. W., and Getzoff, P. C., *J. Urol.*, 1942, **48,** 401.
10. Ormsbee, R. A., Cornman, I., and Berger, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66,** 586.
11. Belkin, M., *J. Pharmacol. and Exp. Therap.*, 1948, **93,** 18.
12. Robbins, M. L., personal communication.
13. Sullivan, B. J., and Wechsler, H. I., *Science*, 1947, **105,** 433.
14. Cornman, I., *Biol. Bull.*, 1947, **93,** 192.
15. Hartwell, J. L., and Shear, M. J., *Cancer Research*, 1947, **7,** 716.
16. Waravdekar, V., and Leiter, J., personal communication. To be published: Abstracts of Fourth Annual Meeting, American Association for Cancer Research, Inc., *Cancer Research*, 1949.
17. Miller, Z. B., Davison, C., and Smith, P. K., *Fed. Proc.*, 1949, **8,** 230.
18. Kalnitsky, G., and Barron, E. S. G., *Arch. Biochem.*, 1948, **19,** 75.
19. Barron, E. S. G., and Singer, R. P., *J. Biol. Chem.*, 1945, **157,** 221.
20. Singer, T. P., and Barron, E. S. G., *J. Biol. Chem.*, 1945, **157,** 241.
21. Barron, E. S. G., Bartlett, G. R., and Miller, Z. B., *J. Exp. Med.*, 1948, **87,** 489.
22. Hollaender, A., Greenstein, J. P., and Jenrette, W. V., *J. Nat. Cancer Inst.*, 1941, **2,** 23.
23. Gjessing, E. C., and Chanutin, A., *Cancer Research*, 1946, **6,** 593.
24. McHugh, J. M., and Ziehl, F. L., *Marquette Med. Rev.*, 1948, **13,** 56, 100 (review of clinical literature).
25. Barron, E. S. G., Bartlett, G. R., Miller, Z. B., Meyer, J., and Seegmiller, J. E., *J. Exp. Med.*, 1948, **87,** 503.
26. Gilman, A., and Philips, F. S., *Science*, 1946, **103,** 409.
27. Kelly, M. G., MacCardle, R. C., and Smith, P. K., personal communication. To be published: Abstracts of Fourth Annual Meeting, American Association for Cancer Research, Inc., *Cancer Research*, 1949.
28. Selye, H., *J. Clin. Endocrinol.*, 1946, **6,** 117.
29. Dustin, A. P., *Ann. bull. soc. roy. sci. méd. nat. Bruxelles*, 1933, Nos. 7–8, 217.
30. Miller, Z. B., Davison, C., and Smith, P. K., data to be published.