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III. Chemotherapy of Tumors in Rats with Certain Ethylenephosphoramides.*† (20381)

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As a continuation of our investigation of the therapeutic effectiveness of ethylenimines in the treatment of transplantable tumors in rats(1,2). we report here results obtained with N,N',N'' - Triethylenephosphoramide, (I), N,N-Diethyl-N',N''-diethylenephosphoramide, (II), N-Pentamethylene-N',N''-diethylenephosphoramide, (III), N-(3-Oxapentamethylene) - N',N'' - diethylenephosphoramide, (IV), N-Tetramethylene-N',N''-diethylenephosphoramide, (V), N,N',N''-*tris* (Tetramethylene)phosphoramide, (VI), N,N', N''-Triethylenethiophosphoramide, (VII) and N - (3 - Oxapentamethylene) - N',N'' - diethylenethiophosphoramide, (VIII).

Materials and methods. The compounds were synthesized in the Pharmaceutical Research Laboratory of The Calco Chemical Division of The American Cyanamid Co. Their structural formulas are shown in Fig. 1. They are colorless, crystalline compounds. (I) melts at 41°C, is extr. soluble in water, very soluble in alcohol, ether and acetone. (III) boils at 95°C/.15 mm Hg and is very soluble in water, benzene, toluene and ether. Both (I) and (III) are very hygroscopic. (IV) melts at 62.5-64°C, is very soluble in water and is soluble in benzene, toluene and ether. (VII) melts at 51.5°C and its solubility in water at room temperature is 19 g in 100 ml. It is soluble in benzene and ether. (VIII) melts at 75-77°C, is slightly soluble in water and very soluble in benzene, toluene and hot petroleum ether. Isotonic saline solutions of the compounds were prepared to contain the desired dose in .2 ml. At first solutions were prepared daily, but equally good results could be obtained with solutions prepared once a week, provided that solutions were kept at about 5°C. One dose was given daily by intraperitoneal injection. In certain cases the subcutaneous route was used for comparison and similar results were obtained. Treatment was given daily throughout the experiment which varied from 2 to 9 weeks. Young male rats, averaging about 150 g were used. In each experiment there were, generally, 4 groups of 6 to 8 rats each; normal controls (N.C.), drug controls (D.C.), tumor controls (T.C.), and tumor treated (T.T.). They were group pair-fed throughout period of treatment. In certain cases the W.B.C. and differential counts were made on all animals in the 4 groups, prior to implantation of the tumor, just before beginning the course of treatment and when the drug was discontinued or the experiment terminated; in certain experiments they were made, also, sometime

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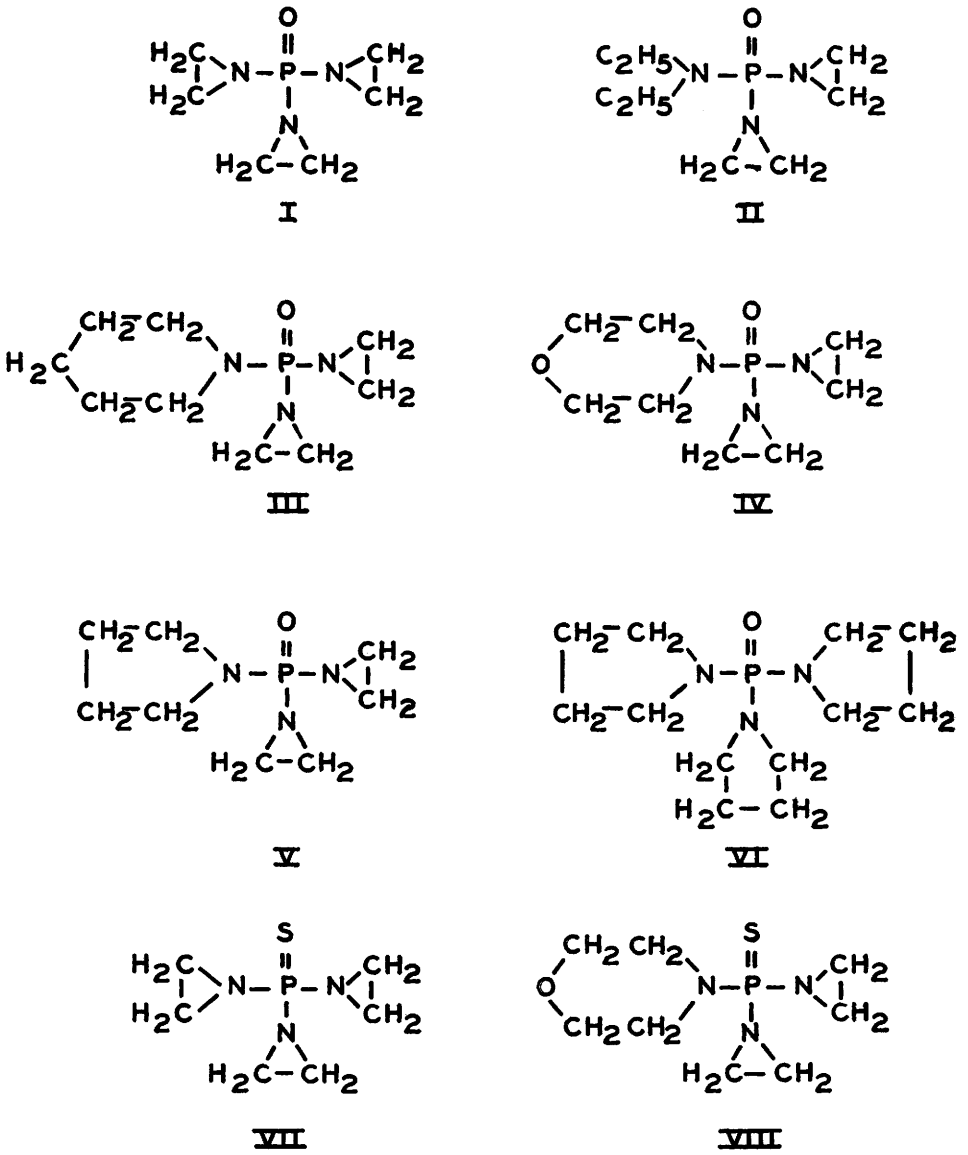


FIG. 1. Structural formulas of products mentioned in the text by numbers.

after the drug treatment was discontinued. The rats were fed the 18% casein diet described by Wase and Allison(3). At the end of treatment the animals in which the tumor had regressed completely were given fox chow, *ad lib.* and examined periodically to find out if they were cured and if they were resistant to growth of further transplants of the tumor. Sarcoma 231 in King A rats, Flexner-Jobling carcinoma and Walker carcinoma 256 in Sprague-Dawley rats and Sarcomas 3 and 7 in Lewis rats were used. We are indebted to Margaret Reed Lewis for the latter 2 tumors which were originally induced by methylcholanthrene in the Lewis rat. The methods for implantation of tumors, measuring growth and determining resistance of cured rats were essentially the same as previously described (1). Groups of rats, treated and controls, were killed at definite periods after transplantation of tumors and certain organs weighed and studied histologically and histochemically.

The weight of organs was expressed in g/100 g of gross body weight.

Results. The initial dose of N,N',N''-Triethylenephosphoramide (I), was the maximum amount which, when injected intraperitoneally, once daily, in normal Sprague-Dawley rats for 7 days, produced no significant leukopenia. Optimum dose of 2,4,6-Triethylenimino-s-triazine for the rat was about .04 mg/kg/D. For (I) it was .43 mg/kg/D. With this dose the W.B.C. of treated rats did not differ materially from that of the control group and there were no manifestations of toxic symptoms. Average increase in body weight was about the same in treated and untreated pair-fed groups.

Preliminary results had shown that ethylenephosphoramides inhibited growth of certain tumors in mice and rats(4-8). Also, they were effective in transplantable mouse leukemia(9).

In 5 experiments with Sarcoma 231 in young King A rats, 150-175 g body weight when treatment began, the tumor regressed completely in 2 of 13 controls and in 26 out of 37 treated. The dose ranged from .4 to .6 mg/kg/D, injected once daily for 2 to 6 weeks. All rats in which the tumor regressed completely were permanently cured. They were challenged several times within a period of 8 to 10 months and remained resistant to growth of grafts. The animals appeared to be similar in well-being to normal controls of their respective groups of same age. Treatment was started when the tumor was well established, usually about 7 days after implantation tumor size averaged 250 to 300 mm^2. In certain experiments treatment was delayed until size of tumor was 800 to 1200 mm^2. In such cases treatment did not begin until about 12 days after implantation. There were no marked differences in number of animals cured or in time required for tumors to regress completely. In all groups treated the tumor continued to grow for a few days at about the same rate as controls, then the majority stopped growing and abruptly regressed with regression continuing to completion. In most cases where the tumors did not regress there was only a slight inhibition in rate of growth followed by acceleration in spite of treatment. Occasionally a tumor which had regressed almost to completion, reversed its course while under treatment and became refractory to the drug.

Compounds (I) and (II) were compared at dose level of .4 mg/kg/D in a few King A rats bearing Sarcoma 231 and preliminary results indicated that this dose, (I) was probably a more effective therapeutic agent than (II). Ten young male King A rats bearing Sarcoma 231 were treated with (III), 5 with .8 mg/kg/D and 5 with 1.2 mg/kg/D. Treatment began 8 days after implantation and continued 18 days. There were 3 regressions in rats getting the smaller dose and 2 with the higher. The 2 treated groups were pair-fed with a group of 5 tumor controls. There were no regressions in the controls and all of the animals died of tumor within 21 days. The King A rat was no longer available and further work with Sarcoma 231 had to be abandoned.

Fifty-eight male Sprague-Dawley rats bearing Flexner-Jobling carcinoma were treated with doses of (I) varying from .30 to 1.26 mg/kg/D. The tumor regressed completely in 42. In 26 tumor controls there was 1 spontaneous regression. There were no significant differences in the number of regressions at the several dose levels. The product was effective over a much wider therapeutic range than T.E.M. Animals that were cured appeared normal except that their testes were smaller than those of controls. Effect on testes varied with dose and length of the period of administration of the product. Results obtained with normal animals receiving same doses were essentially the same as with treated tumor-bearing rats. Weights of testes and other organs in g/100 g of gross body weight are shown in Table I.

Unlike the King A rat, negative takes were not always obtained in Sprague-Dawley rats on first challenge after complete regression of Flexner-Jobling carcinoma. In most cases tumors grew to relatively small size and then regressed spontaneously. In a few instances the tumor grew to a medium size and became static. It was then refractory to treatment. In certain of these cases the tumor was removed surgically and found to be viable. The

TABLE I. Effect of Tumor and Drug on Certain Organs of the Rat.

Rat	Group	Drug	Dose, mg/kg/D	Days treated	% BW change	Wt of organs in g/100 BW: Liver	Thymus	Adrenals	Kidneys	Spleen	Testes	S.V.
						S.D.–F.J.						
NC	(6)*			44		4.0	.17	.01	.73	.21	1.04	.26
TC	(4)			24		5.6	.18	.03	1.07	1.25	1.51	—
DC	(6)	I	.47	45		3.6	.17	.02	.79	.50	.77	.23
TT	(8)	I	.44	45		3.6	.15	.02	.67	.27	.59	.18
"	(6)	I	.85	46		3.7	.14	.02	.67	.41	.36	.28
NC	(11)			45	+ 77	3.5	.16	.02	.65	.25	.94	.17
TC	(6)			40		3.7	.16	.02	.65	.44	1.1	.18
TT	(2)	III	.18	52	+ 58	3.4	.13	.01	.80	.29	.87	.17
"	(3)	III	.37	52	+ 57	3.3	.14	.02	.67	.28	.78	.18
"	(2)	III	.93	52	+ 50	3.9	.10	.02	.72	.26	.62	.15
"	(12)	III	.90	43		3.2	.15	.02	.68	.35	.74	.20
"	(12)	III	.90	43		3.3	.14	.02	.67	.35	.71	.21
"	(3)	III	1.40	52	+ 51	3.5	.11	.02	.78	.28	.48	.22
NC	(6)			45	+ 97	4.4	.16	.02	.75	.28	1.06	.17
TC	(3)			24	+ 29	5.3	.14	.03	.91	1.65	1.20	.09
TT	(4)	IV	.21	51	+ 95	4.0	.12	.02	.86	.29	.90	.18
"	(5)	IV	.43	51	+ 94	4.4	.13	.02	.78	.33	.86	.16
"	(3)	IV	1.00	51	+ 80	4.0	.14	.02	.75	.25	.52	.18
"	(3)	IV	1.67	51	+ 80	3.8	.09	.02	.83	.35	.38	.16
NC	(6)			40		4.6	.21	.02	.80	.41	1.09	.23
TC	(6)			40		4.9	.21	.02	.85	.80	1.11	.19
DC	(4)	V	.75	55		3.0	.17	.02	.67	.19	.57	.23
"	(4)	V	1.01	42		4.8	.17	.02	.66	.46	.74	.18
TT	(4)	V	.94	42		4.5	.18	.02	.80	.53	.70	.16
"	(4)	V	1.09	56		3.4	.15	.02	.59	.38	.44	.21
DC	(4)	VI	1.12	36		4.0	.25	.01	.78	.38	1.09	.24
TT	(4)	VI	.90	36		4.9	.15	.02	.84	1.12	1.07	.12
NC	(4)			53	+ 98	3.7	.13	.01	.73	.36	.92	.21
TC	(4)			51	+ 53	3.8	.11	.02	.71	.59	1.01	.18
DC	(3)	VII	.43	53	+104	3.6	.15	.02	.79	.25	.61	.21
TT	(4)	VII	.33	51	+ 52	3.6	.09	.02	.71	.45	.58	.21
DC	(3)	VIII	.82	53	+109	3.7	.14	.01	.79	.30	.66	.21
TT	(3)	VIII	.64	51	+ 48	3.3	.08	.02	.70	.41	.64	.21
						S.D.–W.256						
NC	(2)			14		3.2	.20	.02	.75	.27	1.18	.16
TC	(3)			14		6.7	.12	.03	.78	1.66	1.18	.08
TT	(8)	VII	.49	29		6.6	.06	.04	.88	1.26	1.03	.11
	not reg											
"	(3)	VII	.47	29		4.0	.11	.02	.82	.42	1.11	.36
	reg											
						L.–S–3						
NC	(4)			16		4.0	.20	.02	.90	.23	1.06	.24
TC	(4)			16		4.7	.18	.01	.84	.59	1.03	.07
DC	(2)	I	.58	9		4.0	.25	.03	.85	.20	1.26	.16
TT	(3)	I	.60	9		4.5	.12	.03	.97	.40	1.26	.05
						L.–S–7						
TC	(3)			17		4.7	.15	.02	.81	.47	1.02	.03
TT	(3)	I	.60	17		4.8	.09	.02	.85	.29	.90	.03

* No. of rats included in the avg results.

rats on recovery from the operation were still susceptible. In other cases the small static tumors were capsules filled with a viscous liquid or cheesy solid containing no viable tumor cells. Once resistance to growth of tumor was established it appeared to be permanent. Animals were challenged periodically up to 1 year after they first proved to be resistant and the results were negative. To ascertain if age of rat influenced development and response to treatment of the tumor 7 adult male rats, averaging about 300 g and bearing the carcinoma were given a dose of .39 mg/kg/D of (I) once daily for 15 days, treatment commencing 10 days after tumor implantation. Complete regressions occurred

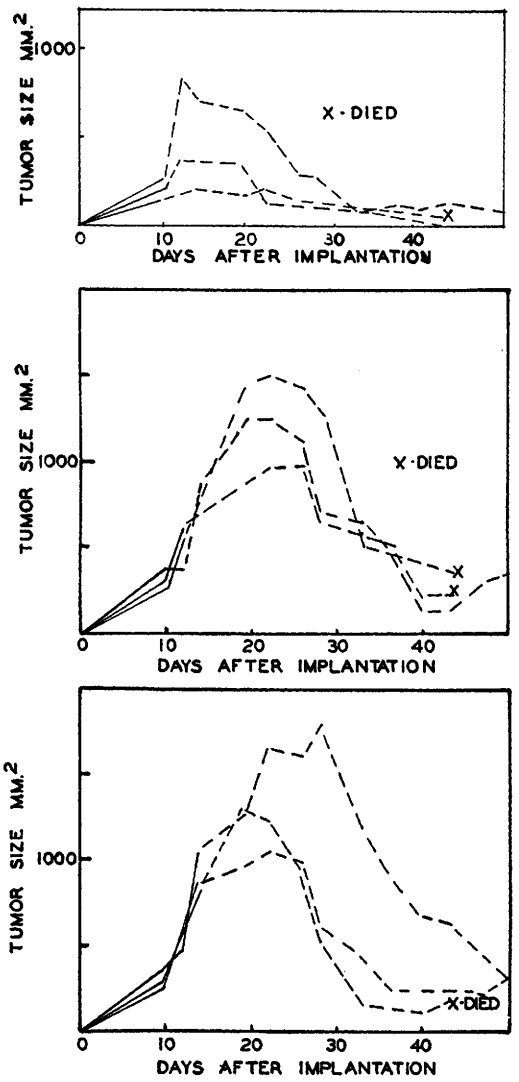


FIG. 2A (top). Growth curves of F-J carcinoma treated with .38 mg/kg/D (I), once daily from 10th day after implantation of the tumor. Period of treatment is indicated by broken lines.

FIG. 2B (center). Growth curves of F-J carcinoma treated with .42 mg/kg/D of (I), once daily from 12th day after implantation. Broken lines indicate period of treatment.

FIG. 2C (bottom). Growth curves of F-J carcinoma treated with (I), from the 14th day after implantation, dose .35 mg/kg/D. Broken lines indicate period of treatment.

in 4 during the treatment and in 2 of the remaining 3 during the 11 days immediately following the course of therapy. No regressions occurred in untreated tumor controls. Similar results were obtained in other experiments using adult rats and it appeared that the age of the rat did not play an important role in the development and regression of the tumor.

In order to determine the effects of treatment at different stages of tumor growth, 16 rats, that had been implanted at the same time

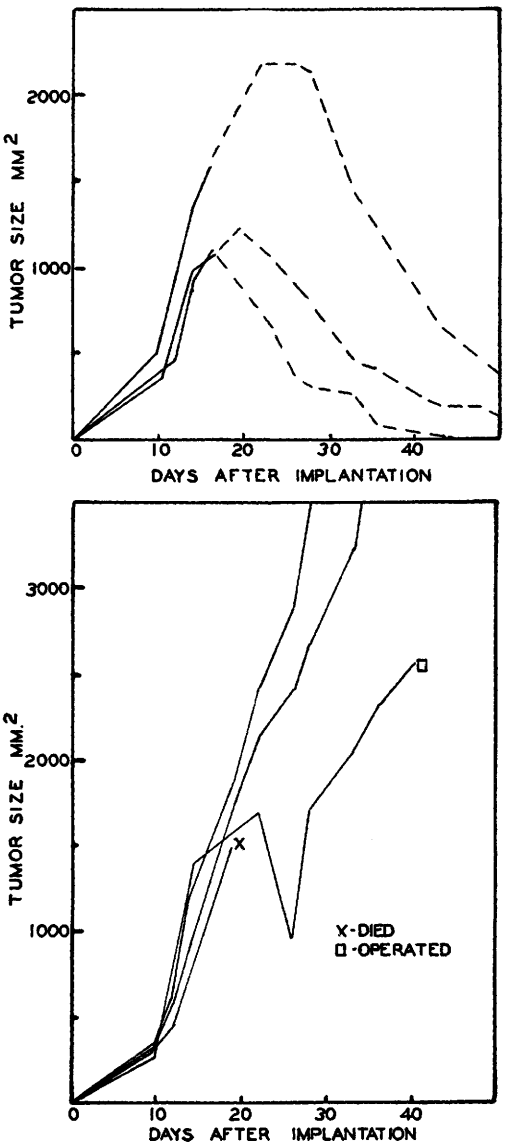


FIG. 2D (top). Growth curves of F-J carcinoma treated with (I) from 16th day after implantation, dose .30 mg/kg/D. Broken lines indicate period of treatment.

FIG. 2E (bottom). Growth curves of F-J carcinoma in group pair-fed untreated controls.

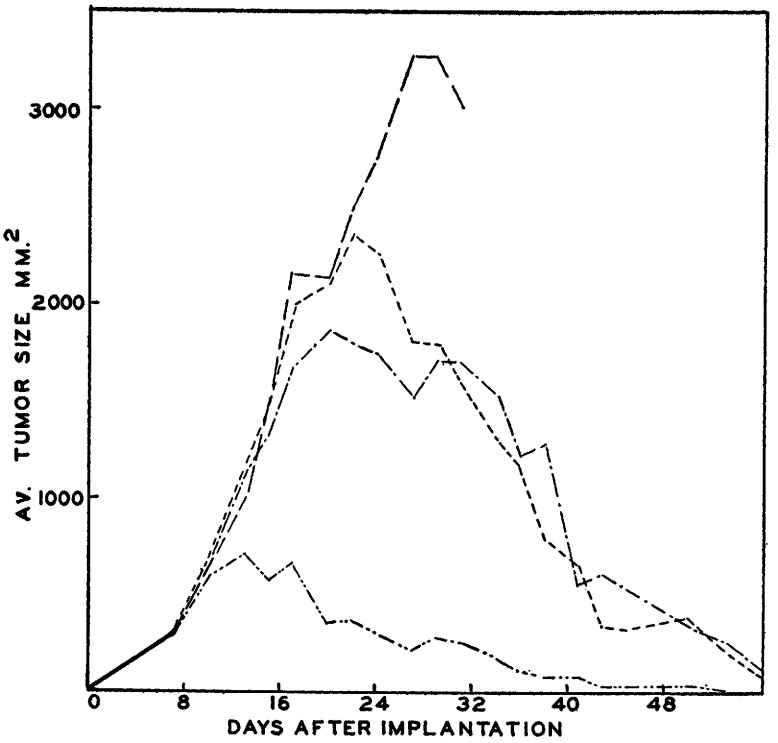


FIG. 3. Average growth curves of F-J carcinoma in pair-fed groups of S-D rats treated with different doses of (IV), once daily. (a) ——, untreated controls; (b) – –, .2 mg/kg/D; (c) —•—, .4 mg/kg/D; and (d) —••—, 1 mg/kg/D. The solid lines indicate period without treatment.

with the carcinoma and having well established tumors were chosen: 4 for controls and 12 for treatment with (I). The 12 animals were divided into 4 groups of 3 each. Therapy was started with the first group 10 days after implantation of tumor; the second, 12 days; the third, 14 days; and the fourth, 16 days. After the first few days of therapy, growth of tumors stopped in all groups and regression set in. This proceeded at a fairly rapid rate at first, particularly in the large tumors, then slowed as tumors became small. There was no significant difference in time required for complete regression of tumors in the rats of the different groups. Comparative behavior of the tumors in the 4 treated groups is illustrated in Fig. 2A-2E.

Fifty-four Sprague-Dawley rats bearing Flexner-Jobling carcinoma were treated with (III) in doses varying from .18 to 1.4 mg/kg/D given once daily for 38 to 53 days. Complete regressions occurred in 36 animals. There was no marked difference in therapeutic effectiveness of the different doses. Higher doses caused greater retardation of tumor growth during the first few days of treatment but the time required for complete regression of the tumor was about the same as with lower doses. Testes weight/100 g body weight decreased with increasing dose. In general, therapeutic range was wider than that of (I). Intraperitoneal and subcutaneous routes were compared at same dose level and results were about the same. With a dose of .74 mg/kg/D there were 5 regressions out of 7 in 53 days in each case. In another experiment, using 24 rats, 12 for each route of administration with a dose of .90 mg/kg/D there were, in 43 days of treatment, 6 regressions in group receiving drug subcutaneously and 8 in that getting it intraperitoneally. In 14 tumor controls there were 2 spontaneous regressions.

Product (IV) produced results similar to those obtained with (III). In 35 treated rats there were 29 complete regressions. It was used in doses varying from .2 to 1.7 mg/kg/D.

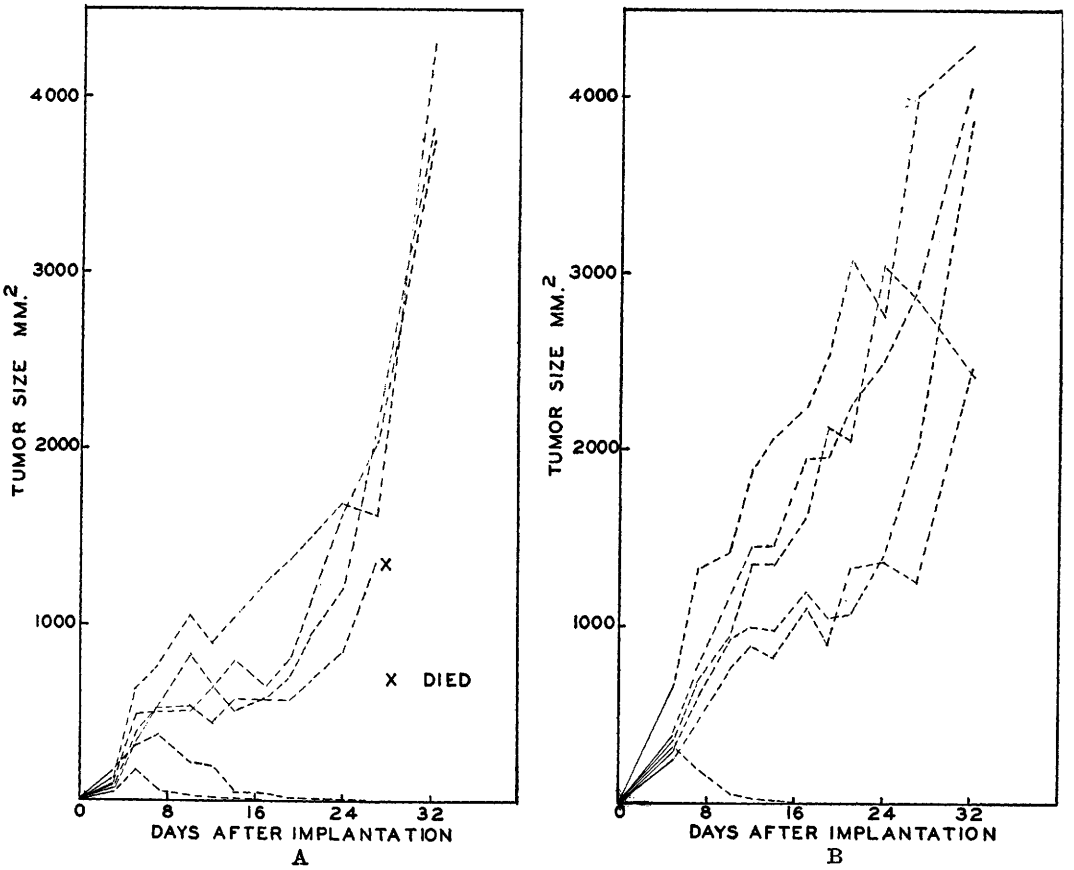
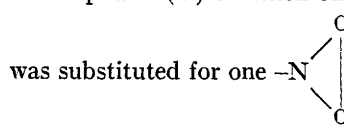


FIG. 4A. Growth curves of Walker 256 tumors in S-D rats, treated with .47 mg/kg/D of (VII). The broken lines indicate period of treatment.

FIG. 4B. Growth curves of Walker 256 tumor in S-D rats, treated with .50 mg/kg/D of (VII). Broken lines indicate period of treatment.

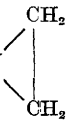
Within this range the dose did not seem to influence significantly total number of regressions. As in other cases mentioned the testes weight dropped with increasing dose. In general, this compound appeared to be the least toxic of the ethylenephosphoramides tested. Average growth rate curves for groups treated with different doses of (IV) are shown in Fig. 3. With the smallest dose the tumor grew during the first week at about the same rate as in untreated animals but after this, growth stopped and regression took place, continuing as in animals treated with higher doses.

Compound (V) in which one azetidyl group



was substituted for one –N behaved like (III) and (IV). There were 8 regressions in 12 treated rats and none in 6 untreated controls. The dose was about 1.0 mg/kg/D.

In (VI), all three



groups were replaced by azetidyl groups. The product was wholly inactive against tumor and did not influence weight of testes.

Products (VII) and (VIII) behaved, in general, like (I) and (IV). Treatment of Sprague-Dawley rats with Flexner-Jobling carcinoma with doses of .50 to .86 mg/kg/D of (VII) once daily gave 11 regressions in 17 treated rats while there were 2 in 14 untreated controls. There were 5 regressions in 8 treated with .94 mg/kg/D of (VIII).

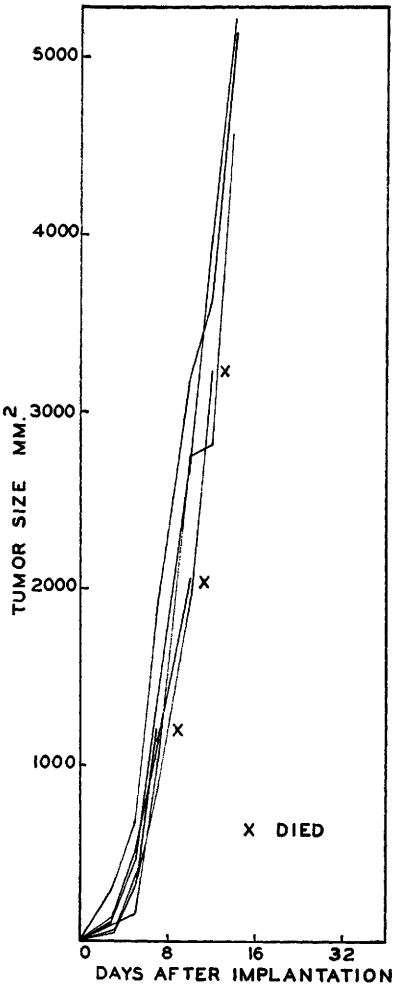


FIG. 4C. Growth curves of the Walker tumor in untreated S-D rats serving as pair-fed controls for 4A and 4B.

Preliminary results with the Walker 256 carcinoma in Sprague-Dawley rats indicated that animals bearing this tumor must be treated soon after implantation to obtain retardation and regression of tumor growth. In 12 rats treated with (VII), 6 were treated with .47 mg/kg/D for 29 days, treatment beginning 3 days after implantation of tumor. There were 2 regressions. The remaining 6 were treated with .50 mg/kg/D for 27 days, treatment beginning 5 days after tumor implantation. There was but 1 regression in this group. It will be seen from growth curves in Fig. 4A-4C that the drug was less effective in retarding growth in the second group than in the first and after the period of retardation growth of the majority of tumors increased in spite of continued treatment.

Fifty-four Lewis rats bearing Sarcomas 3 and 7 were treated with (I), (III) and (VIII) without success. These compounds, used in doses of .52 to 1.6 mg/kg/D, did not retard growth of these tumors. Treatment was started 3, 5 and 7 days after implantation. Tumor growth was about the same in all cases and did not differ significantly from that in untreated controls. The ethylenephosphoramides at the lower dose levels with which good therapeutic results were obtained in Sprague-Dawley rats affected testes only slightly and when the drug administration was discontinued the weight of testes became normal. With the largest doses the testes weight was only about 35 to 40% of normal and did not recover completely when treatment was discontinued. Effect of different doses on weight of testes in a given time is shown for compound (IV) and T.E.M. in Table II.

Action on testes occurs with all of the ethylenimines, in varying degrees. N,N′,N″-Triethylenimino-s-triazine, T.E.M., in relatively large doses, causes testicular damage (10). However, damage is slight with minimum therapeutic doses given to rats for several weeks. The influence of dose of T.E.M. on weight of testes of the Sprague-Dawley rat is shown in Table II. There appears to be a lag period in manifestations of the action of ethylenephosphoramides on both tumor and testes. The length of this period varies with dose of drug. With N,N′,N″-Triethylenephosphoramide (I, T.E.P.A.) at a dose level of .52 mg/kg/D, weight of testes remains normal for 14 to 16 days of therapy and then falls fairly rapidly as treatment continues. This is shown for 3 strains of rats in Fig. 5.

Results of histological and histochemical examination of several tissues as well as effect on pituitary activity will be reported later. Preliminary clinical results with the triethylenephosphoramides were published in two papers recently(11,12).

Growth of transplantable tumors we used was always accompanied by enlargement of spleen and by neutrophylic leukocytosis. Weight of spleen and the extent of neutrophy-

TABLE II. Effect of Dose on Weight of Testes.
(S.D.-F.J.)

Rat	Group	Drug	Dose, mg/kg/D	Days treated	Days off drug	% BW change	g*T/ 100 BW
NC	(4)				63	+138	.99
DC	(3)	IV	.24	64		+168	.84
"	(6)	"	.45	64		+166	.66
"	(6)	"	1.11	66		+142	.43
"	(2)	"	1.76	66		+118	.34
NC	(4)				149	+172	.95
DC	(2)	"	.24	64	85	+156	1.06
"	(1)	"	1.63	66	83	+157	.47
NC	(4)					+112	1.12
DC	(3)	T.E.M.	.04	42		+ 78	1.07
"	(5)	"	.10	42		+ 68	.64
"	(2)	"	.19	15	27	+ 63	.37

* g of testes.

lia corresponded to size of tumor. When tumors regressed completely, by treatment of rats or spontaneously, and rats were cured, both spleen and white blood-cells returned to normal. In those animals not cured of tumor, the spleen remained enlarged and neutrophylia persisted in spite of prolonged treatment. Tumors which did not respond to treatment never regressed spontaneously. Hypertrophy of the spleen and neutrophylia as accompaniment of tumor growth in mice and rats have been reported by others(13,14). Average weights of the spleens of tumor-bearing rats, treated and untreated, in comparison with pair-fed normal groups are given in Table I. The effect of growing and regressing tumors on white blood-cells is shown in Table III. The data express comparative conditions which existed at the time when experiments were terminated.

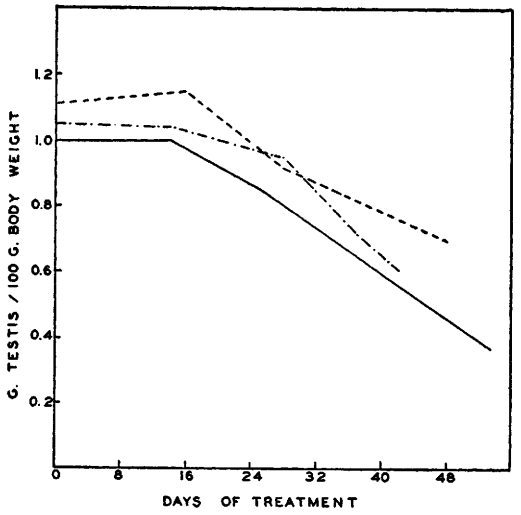


FIG. 5. Average weight curves of testes of S-D, Wistar and Lewis rats given .52 mg/kg/D of (I), and sacrificed in groups of 6 at definite periods of time for comparison with normal groups. The 0 values represent averages for normal groups. The —•—, represents S-D rats; — —, Lewis and the solid curve Wistar rats.

Discussion. Regression of tumors in treated rat did not seem to differ essentially from that which occurred occasionally without treatment and the immunity which resulted was essentially the same in effectiveness and permanence. It would seem probable that some metabolic imbalance helped to initiate a change in the growing tumor which caused it, not only to stop growing, but to regress. This was enhanced by ethylenimine drugs. Tumors which were not affected by the drugs, did not regress spontaneously. Host-tumor relationship must vary considerably. The results did not offer a convincing argument for oncolytic drug specificity.

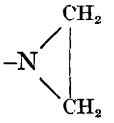
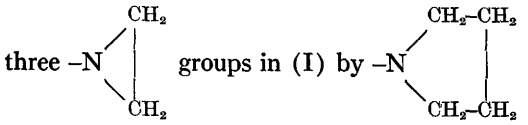
Summary. 1. About 70% of the King A rats with Sarcoma 231 and 72% of the Sprague-Dawley rats with Flexner-Jobling carcinoma were cured by treatment with N,N',N''-Triethylenephosphoramide (I). 2. The replacement of one  group with a piperidyl, or morpholyl or azetidyl group did not affect the oncolytic properties significantly but did reduce the effect on testes; with a dose

TABLE III. Effects of Tumor Growth and Regression on White Blood-Cells of the Rat.

Rat–Tu.	Rat group	WBC	Polys.	Lymph
SD–FJ	NC (19)	18517	15	84
	TC (8)	47736	49	46
	TT reg. (34)	16012	19	79
	TT unreg. (11)	42133	50	47
KA–231	NC (6)	18550	24	72
	TC (6)	75000	68	28
	TT reg. (6)	12483	28	70
	TT unreg. (4)	63875	74	23
L–S-3	NC (6)	18567	19	80
	TC (6)	32800	40	56
	TT (9)	29833	52	47
L–S-7	TC (6)	33983	54	45
	TT (6)	27400	52	46

of .85 mg/kg/D of (I) given once daily for 45 days weight of testes of Sprague-Dawley rat was only about 35% of that of the normal controls, with a similar dose of (III) given in the same way for the same time it was about 79% of normal. Similarly, with a dose of .44 mg/kg/D of (I), once daily for 45 days the weight of the testes was 57% of normal while with a similar dose of (IV) given for 51 days weight of testes was 81% of normal. 3. The Flexner-Jobling tumor in the Sprague-Dawley rat regressed completely in about 70% of the animals treated with (III) and in about 80% of those treated with (IV). 4. At doses of about .2 mg/kg/D of (III) and (IV) good therapeutic results were obtained and weight of testes was but slightly below normal; when drug treatment was discontinued testes weight became normal. 5. The substitution of all



groups resulted in compound (VI) which was inactive. 6. Compound (V) behaved like (III) and (VII), and (VIII) like (I) and (IV). 7. T.E.M. at a dose level of .04 mg/kg/D, cured 75% of the rats treated and weight of testes was normal. 8. Spleen enlargement and neutrophylic leukocytosis accompanied growth of all tumors studied. Animals recovered from both conditions when tumors regressed completely. 9. Sarcomas 3 and 7 in the Lewis rat did not respond to treatment with any of the ethylenephosphoramides nor with T.E.M. 10. The Walker tumor in the Sprague-Dawley rat responded to treatment better when therapy was started early; 3 days after implantation instead of later.

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