TABLE I. Eosinopenia during the Last Third of Pregnancy in the Mice Investigated.

Stock	Type	No. of mice	Mean No. of eosinophils	Difference	t	P_t
	Section I. Samples obtained from 08:30 to 13:00					
Zb	NP	4	133	73	2.598	.026
	P	8	60			
Z	NP	8	121	47	1.289	.217
	P	9	74			
Ax	NP	5	518	428	2.966	.021
	P	4	90			
A	NP	4	164	32	.923	.375
	P	10	132			
	Section II. Samples obtained from 22:00 to 02:30					
Zb	NP	7	66	51	2.481	.029
	P	7	15			
Z	NP	4	80	42	1.023	.333
	P	7	38			
Ax	NP	9	212	111	2.837	.011
	P	10	101			
A	NP	6	174	53	2.966	.260
	P	8	121			

NP = nonpregnant; P = pregnant.

partum(2).

Summary. Eosinopenia was observed during the last third of pregnancy in several stocks of mice. The mean eosinophil level of females of the Ax and the Zb stock, pregnant 2 weeks and more, was less than half of that noted in non-pregnant mice. Eosinopenia was also noted during the 48 hours following the delivery of the young.

The authors are indebted to Dr. Maurice B. Visscher for advice, to Dr. John J. Bittner for the mice employed and to Miss Ruth O. Vrudny for valuable assistance.

1. Halberg, F., Bittner, J. J., and Visscher, M. B., *Blood,* 1951, v6, 832.

2. Davis, M. E., and Hulit, B. E., *J. Clin. Endocrinol.,* 1949, v9, 714.

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Serial Propagation *in vitro* of Agents Producing Inclusion Bodies Derived from Varicella and Herpes Zoster.* (20354)

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(With the technical assistance of Helen M. Witton.)

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It is generally accepted that serial propagation in the laboratory of the agents of varicella and of herpes zoster has not heretofore been accomplished. Certain of the earlier published reports regarding their serial propagation have more recently been attributed to possible confusion with viruses pathogenic for lower animals. However, morphological evidence has suggested that a single passage of the agents of varicella and herpes zoster has been achieved. Thus Rivers(1,2) observed focal lesions with intranuclear inclusions in the monkey testicle following the local inoculation of varicella vesicle fluid. Goodpasture and Anderson(3) grafted human skin on the

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TABLE I. Isolation of Cytopathogenic Agents in Tissue Cultures Inoculated with Varicella Vesicle Fluid.

Patient	Age	Day illness vesicles aspirated*	Vesicle fluid frozen before use	Isolation in roller tubes†	Day cult. C.P.‡ first observed
McE.	23	4	24 mo	3/3	8
Wel.	4	2	22 "	3/4	8
Rud.	7	2	9 "	3/4	6
And.	8	1	1 "	2/4	6
Dru.	15	3	2 wk	3/4	7
Pat.	4	1	1 "	1/4	13

* Dated from appearance of vesicles.
† No. cultures showing foci/No. inoculated.
‡ Cytopathic changes.

chorioallantoic membrane of the chick embryo. Although negative results were obtained following inoculation of varicella vesicle fluid, in one experiment histological examination of grafts following inoculation with fluid from herpes zoster lesions revealed intranuclear inclusions. Using essentially the same technic, Blank, Coriell and Scott(4) likewise in one instance demonstrated inclusion bodies after inoculation of zoster material.

In 1948 we began an investigation of the potentialities of suspended cell cultures of human tissues as a medium for the isolation of the causative agent of varicella. Eosinophilic intranuclear inclusions were demonstrated on examination of tissue fragments removed from the cultures at intervals after the introduction of varicella vesicle fluid(5). Efforts to maintain the responsible agent on serial passage in this type of culture were unsuccessful. More recently roller tube cultures of human tissues have been applied to the same objective. The results so far obtained are here reported in a preliminary manner. As will be shown it has been possible to isolate and maintain in serial passage cytopathogenic agents apparently derived from specimens of varicella vesicle fluid as well as others apparently obtained from herpes zoster vesicle fluid.

Materials and methods. Vesicle fluid specimens were collected and stored as previously described(5). Roller tube cultures were prepared as in our studies on poliomyelitis(6) utilizing either human embryonic skin-muscle tissue or foreskin tissue obtained from boys between the ages of 3 months and 3 years. In the present experiments the nutrient fluid for the cultures consisted of bovine amniotic fluid (90%), beef embryo extract (5%), horse serum (5%), antibiotics, soybean trypsin inhibitor and phenol red as recently described (6,7). Changes of the nutrient fluid were made at 3- or 4-day intervals. In certain experiments for histologic examination tissues were grown on coverslips coated with chicken plasma that were placed in roller tubes; these preparations were fixed with Zenkers-acetic acid and stained with hematoxylin and eosin.

Experimental. 1) *Studies with varicella vesicle fluid.* Vesicle fluids derived from 11 cases of varicella were inoculated individually, usually in the form of 0.1 ml aliquots of a suspension in milk, into groups of roller cultures of human embryonic skin-muscle tissue. The exact concentration of the fluid in milk cannot be stated. In every instance tissue growth was well established at the time of inoculation. As summarized in Table I, in cultures inoculated with fluids from six of these cases, focal cytopathogenic lesions of a characteristic appearance developed which were readily seen on microscopical examination of the living cultures. These lesions usually were first observed from the 6th to 8th day after inoculation. They consisted of small collections of swollen, rounded refractile cells which contrasted sharply with the surrounding fibroblastic or epithelial outgrowth. Those foci of affected cells developing in the sheets of cells of normal appearance increased slowly in size. The cells in the center of such focal areas gradually degenerated over the course of several days, while slow peripheral extension of the lesion continued for days or weeks as contiguous cells became infected. Study of stained coverslip preparations from

TABLE II. Serial Propagation of Two Agents Isolated following Inoculation of Varicella Vesicle Fluid in Cultures of Human Tissue.

			McE. strain		Wel. strain	
Passage	Tissue	Cumulate days in culture	Day C.P.* noted	No. cult. C.P. / No. cult. inoc.	Day C.P. noted	No. cult. C.P. / No. cult. inoc.
1	Emb. S-M†	0	8	3/3	8	3/4
2	"	9	3	3/3	4	2/3
3	Foreskin	20	10	1/2	5	2/2
4	"	34	5	8/9	5	10/16
5	"	45	4	3/4	3	3/3
6	Emb. S-M	58	3	15/15	3	15/15
7	"	67	3	9/10	3	10/10
8	"	81	3	8/8	3	8/8
9	"	94	3	6/6	3	6/6
10	Foreskin	124	4	5/6	4	6/6

* C.P. = cytopathic changes.
† Emb. S-M = human embryonic skin and muscle tissue.

roller tube cultures inoculated with vesicle fluid material has shown that the changes in cellular morphology are characteristically associated with the presence of inclusion bodies. At the margin of a focus occasional cells may be observed that show no gross morphological changes, yet contain small granular intranuclear inclusions. Rounded and swollen cells almost invariably possess intranuclear eosinophilic inclusions. Occasionally the larger swollen cells are multinucleate, with each nucleus containing an inclusion; such cells resemble the multinucleate giant cells described by various workers in the lesions of varicella, herpes zoster and herpes simplex(8). Focal lesions developing in a pre-existing loose network of cells progress irregularly, but manifest similar changes. The morphological changes observed in the cultures will be reported in more detail in a future communication.

All six of the agents isolated following the inoculation of varicella fluids have been propagated serially in tissue culture as indicated by the successive development in subcultures of focal areas of cellular enlargement and degeneration. Two of the strains (McE. and Wel.) have now been maintained for 10 passages as summarized in Table II. The inoculum employed to initiate the third and succeeding passages in these experiments consisted of 0.1 ml of a suspension of coarsely ground tissue removed from the preceding set of cultures. On one occasion, the infected tissue suspension was frozen in the CO_2 box prior to use; otherwise, the inoculum was prepared on the day it was employed. Fig. 1 to 3 depict representative cytopathic changes observed during the serial propagation of the McE. and Wel. agents. In contrast to the results obtained with tissue suspensions, attempts to establish passages with inocula consisting of centrifuged fluids removed from the infected cultures have so far been unsuccessful.

Studies designed to elucidate the nature and etiological relationship of these cytopathogenic agents thus isolated to varicella are in progress. In one experiment, vesicle fluid material of known infectivity for cultures did not produce cytopathic changes when inoculated following heating at 60°C for 30 minutes. In none of more than 70 control cultures maintained during the passage experiments have focal lesions been observed; these tubes routinely received inocula consisting of a suspension of tissue derived from control cultures of the preceding passage. Tissue suspensions infective for cultures have been without obvious effect when inoculated into suckling mice or into the developing hens egg by various routes. Attempts at *in vitro* neutralization of the cytopathogenic effect with convalescent serum from cases of varicella have so far been unsuccessful. It is possible that the close association of the infective agents with cell constituents may be involved in this apparent lack of neutralizing effect. In certain preliminary experiments in which culture fluids harvested from tubes showing cytopathic changes have been employed as antigen in

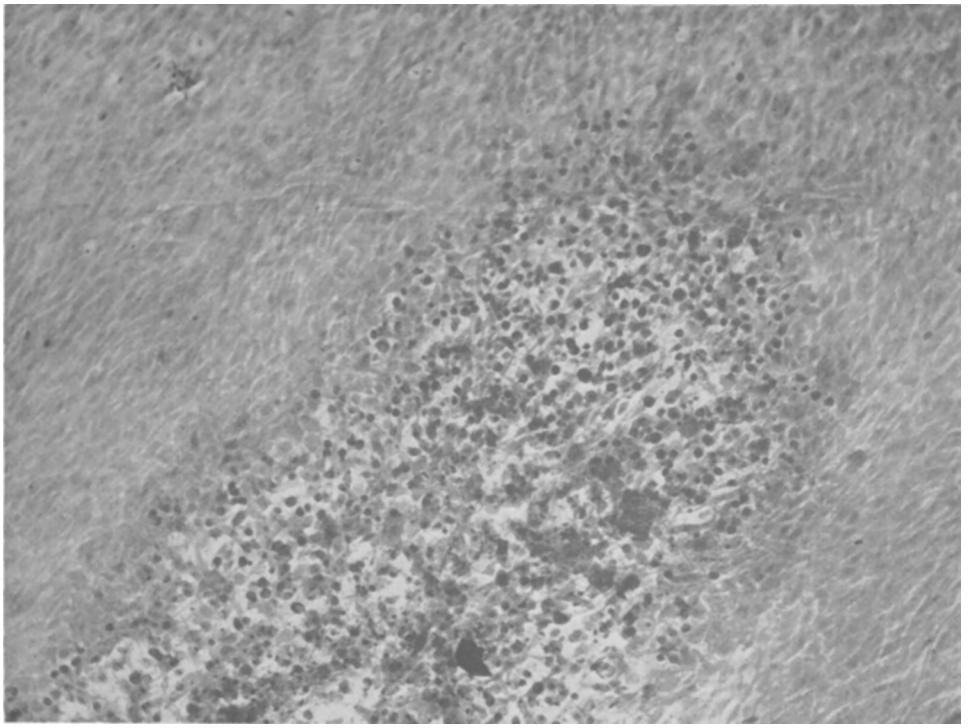

FIG. 1. Varicella. McE. strain; 3rd tissue culture passage. Focal lesion in foreskin culture; 7th day after inoculation. (H & E × 100).

complement fixation tests on paired serum specimens obtained from cases of varicella, a rise in antibody titer has been observed. The specificity of this reaction is under investigation.

2) *Studies with herpes zoster vesicle fluid.* Similar focal cytopathic changes have been observed in cultures inoculated with material obtained on the day of appearance of vesicles from an 80-year-old (Sto.) with thoracic herpes zoster† and with fluid collected from a 30-year-old woman (Pie.) with involvement of the ophthalmic branch of the trigeminal nerve. The *Sto.* inoculum had been stored in the frozen state for 21 months prior to use, while the *Pie.* fluids were inoculated on the day of collection. Serial propagation in tissue culture of these two agents has been accomplished, as summarized in Table III, again with inocula consisting of ground tissue suspensions. (These manipulations have been performed in a separate room from that in which the varicella agents have been maintained.) The cytopathogenic effect observed in the cultures closely resembles that obtained following the introduction of varicella vesicle fluid. Examination of stained preparations from the second tissue culture passage of the *Sto.* strain has also revealed that the rounded swollen cells composing the focal lesions contain intranuclear inclusions (Fig. 4). Tissue suspensions prepared from cultures of the *Sto.* and *Pie.* agents, that were successfully employed to initiate culture passages, have produced no overt symptoms in newborn mice when inoculated by various routes.

Discussion. Inoculation of roller tube cultures of human tissues with materials derived from the eruptive lesions of varicella and of herpes zoster has revealed the presence of cytopathogenic agents capable of producing intranuclear inclusions. These, subsequently, have been maintained in serial passage. A peculiarity of their behavior in the culture system employed has been the apparent failure of infectious material to appear in the fluid phase. The focal lesions appear to in-

† Collected by Dr. F. C. Robbins.

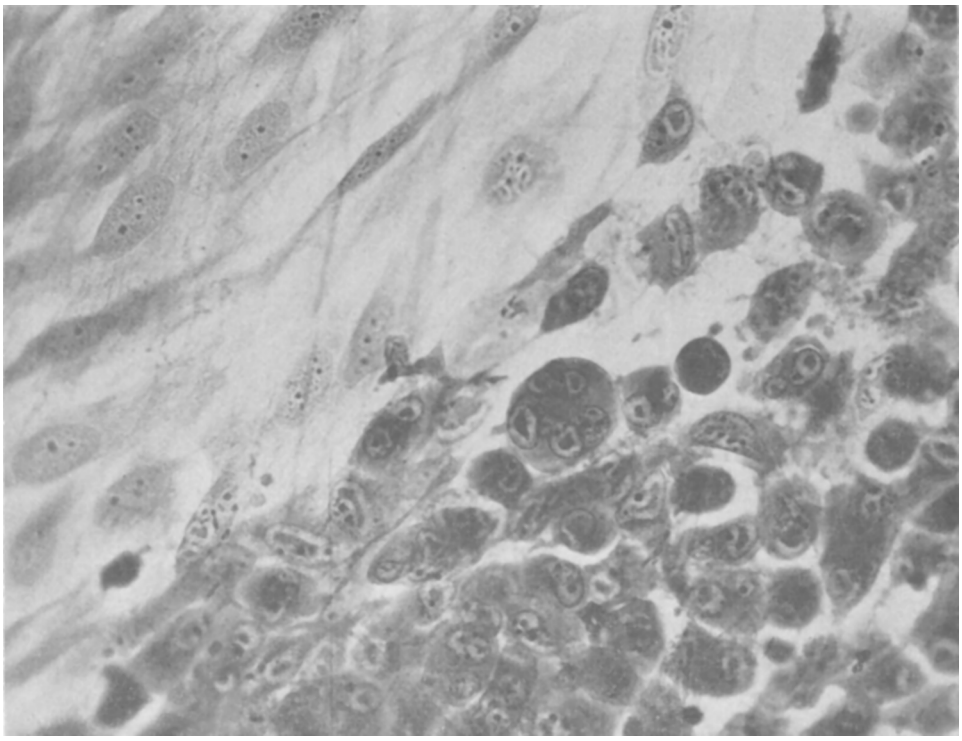

FIG. 2. Varicella. Wel. strain; original passage in human embryonic skin-muscle tissue. Edge of focal lesion on 6th day after inoculation of vesicle fluid. Note intranuclear inclusions and multinucleate cells. (H & E × 550).

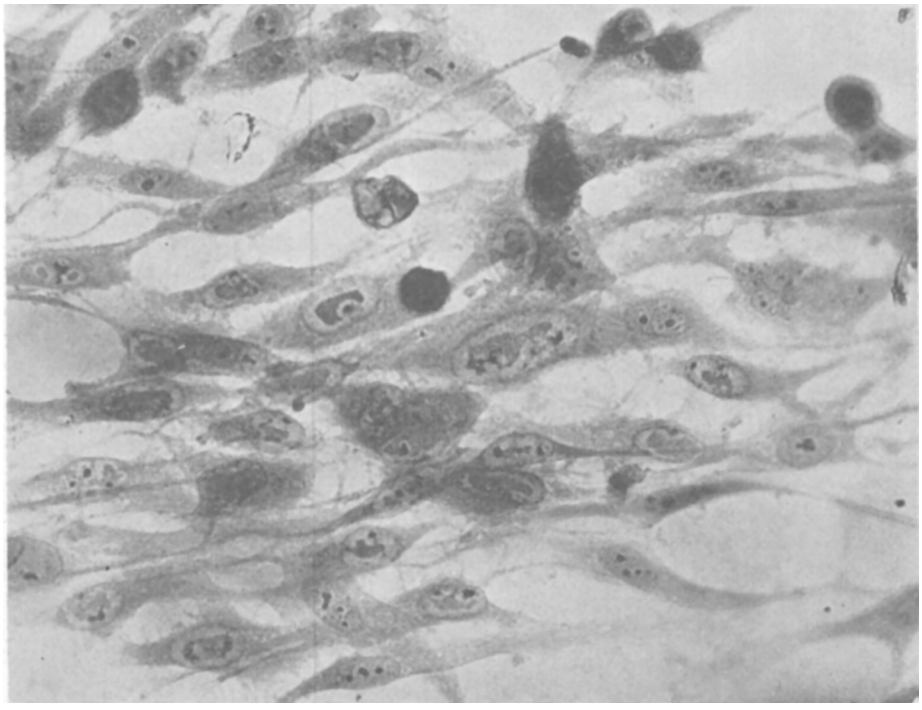

FIG. 3. Varicella. Wel. strain; 5th culture passage in foreskin tissue. Focus of cells with intranuclear inclusions on 6th day after inoculation. (H & E × 550).

TABLE III. Serial Propagation of Cytopathogenic Agents Isolated following Inoculation with Herpes Zoster Vesicle Fluid.

	Sto. strain				Pie. strain			
Passage	Tissue	Cumulate days in culture	Day C.P.* noted	No. cult. C.P. / No. inoc.	Tissue	Cumulate days in culture	Day C.P. noted	No. cult. C.P. / No. inoc.
1	Emb. S-M	0	6	2/2	Foreskin	0	5	3/6
2	"	15	5	10/10	"	14	3	6/6
3	Foreskin	36	3	6/6	Emb. S-M	44	5	2/3
4	"	66	3	5/6				

* C.P. = cytopathic changes.

crease in size by infection of immediately adjacent cells. On prolonged cultivation, however, an increase in the number of focal lesions occurs. In one experiment in which infected cultures were maintained for 8 weeks approximately 90% of the proliferating tissue became involved. Even so, extension of the lesions was continuing at the end of this period. The significance of these observations is being investigated. It is at present clear, however, that in the utilization of tissue culture technics for the isolation of unknown agents, consideration must be given to passage of tissue suspensions, as well as to the passage of fluid inocula.

No evidence has been obtained that the agents isolated are not those responsible for varicella and herpes zoster. Yet no definitive statement can now be made regarding their nature, their etiological relationships, or the interesting question of the possible identity of the agents of herpes zoster and varicella. It is to be noted that the virus of herpes simplex when propagated in tissue cultures of the type here employed manifests an early focal tendency to induce lesions but then rapidly

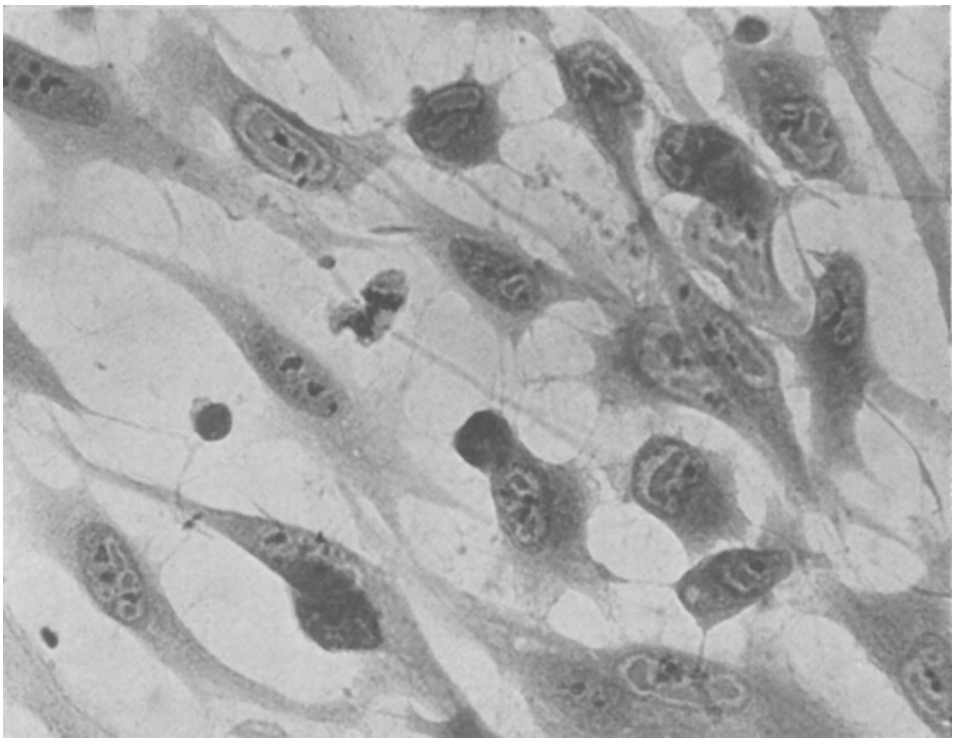

FIG. 4. Herpes zoster. Sto. strain; 2nd culture passage in human embryonic skin-muscle tissue. Edge of focal lesion with intranuclear inclusions on 12th day after inoculation. (H & E × 825).

brings about a widespread infection of the cell population resulting in an appearance quite dissimilar to that here described(9). The failure of these agents to produce obvious infection of suckling mice, in view of the high degree of susceptibility of this animal to the virus of herpes simplex(10), also indicates that they are not to be identified with the latter. We have no indication that a Rickettsia-like agent of the type described by Sprunt and Hirst(11) was present in our cultures.

Summary. The inoculation of roller tube tissue cultures of human tissues with vesicle fluid derived from patients with varicella has resulted in the isolation of six cytopathogenic agents. Two of the strains have been maintained for 10 tissue culture passages by employing tissue suspensions as the passage material. Histologically the lesions produced consist of focal accumulations of cells which become swollen, and then degenerate; characteristically, such cells contain intranuclear inclusion bodies. From the eruptive lesions of two cases of herpes zoster, cytopathogenic agents of a similar nature have been isolated and have been propagated serially in cultures of human tissue.

1. Rivers, T. M., *J. Exp. Med.*, 1926, v43, 275.
2. ———, *J. Exp. Med.*, 1927, v45, 961.
3. Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1944, v20, 447.
4. Blank, H., Coriell, L. L., and Scott, T. F. M., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 341.
5. Weller, T. H., and Stoddard, M. B., *J. Immunol.*, 1952, v68, 311.
6. Robbins, F. C., Weller, T. H., and Enders, J. F., *J. Immunol.*, 1952, v69, 673.
7. Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 100.
8. Blank, H., Burgoon, C. F., Baldridge, G. D., McCarthy, P. L., and Urbach, F., *J. A. M. A.*, 1951, v146, 1410.
9. Enders, J. F., and Robbins, F. C., unpublished observations.
10. Kilbourne, E. D., and Horsfall, F. L., *J. Immunol.*, 1951, v67, 321.
11. Sprunt, K., and Hirst, G. K., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 178.

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Antagonism of Toxicity of Vanadium by Ethylenediamine Tetra Acetic Acid in Mice. (20355)

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It has been shown by a number of workers (1-3) that ethylenediamine tetra acetic acid (EDTA) forms complexes with many metals. This principle has been applied, to a very limited extent, to experimental lead and cobalt poisoning in animals(4-6).

Only occasional references to antidotes for vanadium are to be found in the literature. Priestly(7) found that potassium ferrocyanide and substances containing tannin were effective antidotes for vanadium. BAL on the other hand, according to Sjoberg(8), was not only ineffective in reducing vanadium toxicity by inhalation in rabbits, but actually worsened the effect.

In view of the peculiarly strong metal-complexing potentialities and low toxicity of EDTA, and the possible need for an effective antidote in acute cases of vanadium poisoning, the prophylactic value of EDTA was tested in vanadium-poisoned mice. This study constitutes a part of a larger program on vanadium toxicology currently in progress in these laboratories.

Materials and methods. The calcium disodium salt of EDTA* was used for this study, because it has been shown(9) that the sodium salts of this compound injected into an animal

* This salt was prepared by combining millimolecularly equivalent amounts of Na_2EDTA (Eastman) and $CaCl_2$. Grateful acknowledgement is made of the generous contribution of a solution of $CaNa_2EDTA$ by the Bersworth Chemical Co., used for part of this study.