hibits growth by interfering with protein synthesis. This interference was seen morphologically when the blastema was formed. It may be that the analogue prevented growth of already formed cells, or it may have acted by cutting down the number of blastema cells. Externally the only sign of this inhibition of protein snythesis was retardation of growth, and the exact mechanism remains unknown.

No specific morphological abnormalities were encountered in the animals treated with N-dichloroacetyl-dl-serine. The 2 early stages of the regeneration process (*i.e.* the wound-healing and dedifferentiation phases) were not affected. But by the 25th day the growth of the regenerates in the analogue-treated animals was lagging behind that of the controls. Later there was also a slight delay in digit differentiation. Thus it seems that the process of regeneration was affected in the later stages, when the blastema had formed and growth and differentiation had begun.

Summary. The effect of N-dichloracetyl-dl-serine, when injected into regenerating forelimbs of salamanders, was observed. Regeneration was studied over a period of 56 days. Except for a decrease in growth of the regenerates no significant morphological abnormality was produced. A 0.2% solution of the substance proved more effective than a 0.1% concentration. The substance affected the regenerating tissues at the later phases of regeneration, namely active growth and differentiation.

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Received October 18, 1963. P.S.E.B.M., 1964, v115.

Interaction of Mycoplasma (PPLO) and Murine Lymphoma Cell Cultures: Prevention of Cell Lysis by Arginine.* (28871)

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Preliminary characterization of several strains of mycoplasma that are cytocidal for L5178Y and P388 murine lymphoma cell lines has been presented(1). These strains were isolated from various types of cell cultures, but produced no discernible cytopathology in cell cultures other than the murine lymphoma cell lines. Many other mycoplasma strains such as T-5, Eaton agent, and other strains isolated from cell cultures were incapable of adversely affecting L5178Y cells carried *in vitro*.

In the earlier report, there were 3 indications that the mechanism of lysis of the L5178Y cells might not depend upon infection of the cells: first, yield of mycoplasma following inoculation of various types of cell cultures was not related to occurrence of cytopathology; second, time of lysis of a L5178Y cell culture was correlated with growth curve of the mycoplasma rather than with multiplicity of infection; finally, L5178Y cells grown in the ascites form in genetically susceptible mice were completely resistant to the lytic mycoplasma, even when the mycoplasma and cells were mixed at high multiplicity and preincubated prior to intraperitoneal injection.

The present paper reports results which indicate that the cytopathic effect produced by these lytic strains is independent of cellular infection and is, in fact, related to production of mycoplasmal "toxins" that deplete the medium of arginine.

Materials and methods. The basic techniques used for handling mycoplasma and L5178Y cells have been described(1). Briefly, the L5178Y cells are carried *in vitro* in "L-1"

* Supported (in part) by USPHS Training Grant from Inst. of General Med. Sciences.

medium with added calf serum (5%) and, in most cases, penicillin and streptomycin. The lytic mycoplasma strains, designated by names such as "12-3," "L.M.-2" are titered either in L5178Y cultures as endpoint $TCID_{50}$/ml = lytic units or on "PPLO agar" as colony-forming units/ml (CFU/ml). The two methods give good agreement for the 12-3 strain but some strains, such as L.M.-2, grow poorly on our agar. In the case of the 12-3 strain, agar titrations are done with replicate plating of 0.01 ml quantities of serial 10-fold dilutions, then counting the colonies after 3 days on plates having between 10 and 200 colonies per spot.

Ultrafiltrates of lysates of infected L5178Y cultures and ultrafiltrates of extracts of PPLO agar minces were both prepared by filtration through VM Millipore filters (pore size: 50 mμ $\pm$ 3 mμ). Previous work in both this laboratory(1) and others(2) indicates that such filters are reliable sterilizing devices for mycoplasma. Preparations were first clarified by centrifugation in a Serval centrifuge for one hour at 13,000 rpm. The integrity of the filter was checked in every experiment that involved either an ultrafiltrate or a U-tube. This was done by repeated sampling of such preparations, inoculation of L5178Y cultures and PPLO agar dishes, and blind passage of negative L5178Y cultures every 2 or 3 days for at least 3 times.

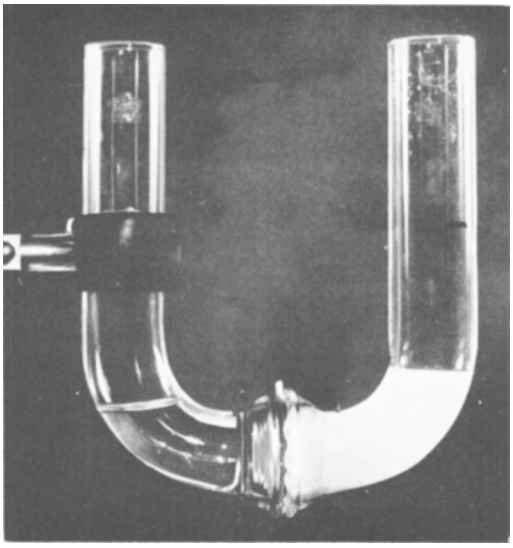

FIG. 1. U-tube with medial VM Millipore filter barrier. A suspension of 88 mμ latex particles was poured into the right arm to level of black line. After 16 hr the photograph was taken to demonstrate the clarity of filtrate in left side arm.

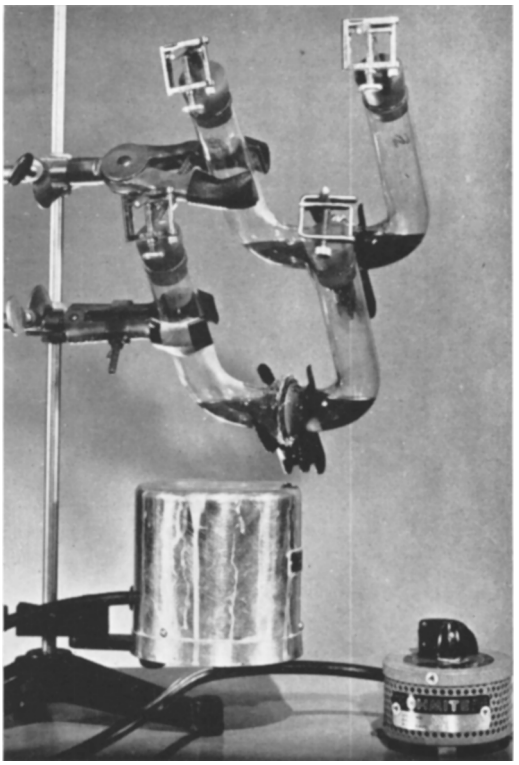

FIG. 2. U-tubes as set up for lysis experiments. Stopper clamps are opened before stoppers are removed or replaced in order to prevent any pressure differentials in the 2 arms. Magnetic stirrers are used in all arms to enhance diffusion across membrane.

Viable counts of L5178Y cells were done with a hemocytometer using cell suspensions diluted 1:1 with 0.5% trypan blue in phosphate buffered saline.

Results. U-tube† *experiments.* A direct demonstration that the cytocidal effect of the lytic mycoplasma strains did not require cellular infection was achieved with L5178Y cultures grown in U-tubes. The two arms of each U-tube were separated by a VM Millipore membrane filter, the apparatus being held together with a special clamp (Fig. 1, 2). In each experiment reported here, where a single arm was inoculated with mycoplasma, samples were repeatedly obtained from the

† Made by Kontes Glass Co., Vineland, N. J.

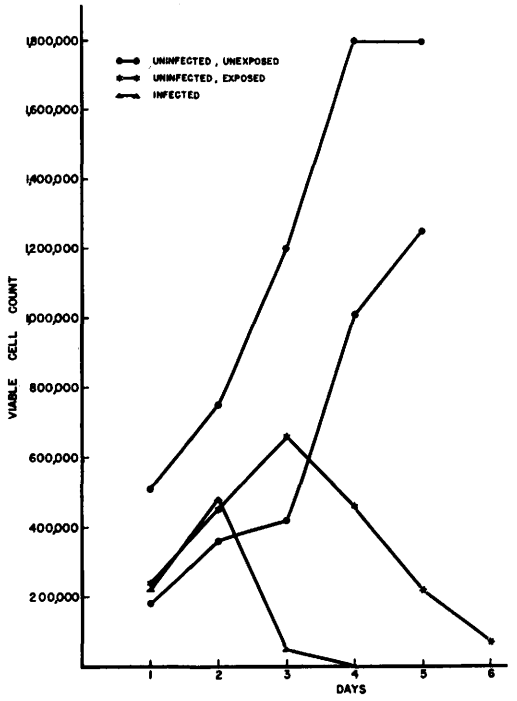

FIG. 3. Growth curves of L5178Y cells in U-tubes. Each curve represents the results from one arm of a U-tube. Uninfected, exposed arm was contralateral to infected (with 12-3 strain) arm of one U-tube, while the 2 uninfected, unexposed curves are from a U-tube with similar cultures in both arms.

contralateral side, and sterility ascertained by blind passage in L5178Y cultures and by inoculation onto "PPLO" agar dishes.

The results of a typical experiment using this basic technique (Fig. 3) show that U-tube cultures, when both arms are uninfected, allow progressive cellular growth to plateau counts of approximately 1.8×10^6 cells/ml; when one arm is infected with lytic mycoplasma, lysis of the infected arm is followed after a lag of about 24 hours by lysis of the noninfected contralateral culture.

L-1 medium alone neither supports replication nor prevents thermal inactivation of lytic mycoplasma when such medium controls are incubated at 37°C(1). Hence, the demonstration of lysis without infection does not of itself exclude the possibility that the relationship between L5178Y cells and mycoplasma in an infected culture is a "special" one. For instance, it could be envisaged that when these lytic mycoplasma infect L5178Y cells, a toxic by-product is produced. When other types of cell cultures are infected, no such by-product would be produced and, therefore, no cytopathology would result. Consequently, U-tube experiments were done in which the infected arm contained L-1 medium and mycoplasma growing under circumstances other than in conjunction with L5178Y cells. The uninfected arm contained a growing L5178Y culture as before. Fig. 4 presents growth curves of noninfected contralateral arms of U-tubes where the circumstances of mycoplasma growth are: 1) hamster embryo cell cultures contaminated with the 12-3 strain of mycoplasma but without evident cytopathology; and 2) minced agar containing numerous small mycoplasma colonies suspended in L-1 medium. Samples from the infected arms titered on PPLO-agar indicated that continued growth of the mycoplasma occurred during the 4-day period of the experiments.

These results demonstrate that growth of lytic mycoplasma in hamster embryo cell cultures that are not adversely affected, or even growth in a cell-free situation, results in the same type of deleterious effect on the contralateral L5178Y culture, as growth of these agents in L5178Y cultures. The possibility that production of the lytic factor is related to a special relationship between L5178Y cells and mycoplasma is therefore excluded. The possibility is not excluded that the difference between L5178Y cells and other cells is related to differences in susceptibility to the lytic factor.

The failure of medium controls to support replication of these mycoplasma implies that the L5178Y cells are growth promoters in infected L5178Y cultures. Whether or not intracellular infection is necessary for this action was tested with U-tube experiments in which the infected arm contained mycoplasma suspended in L-1 medium without cells. The contralateral arm either contained L5178Y cells growing in L-1 medium or L-1 medium alone. The infected arms were sampled at various times and titered for colony forming units of mycoplasma. These results (Table I) show that intracellular in-

TABLE I. Mycoplasma Growth Promotion by L5178Y Cells Without Cellular Infection.

PPLO in L-1 medium*	Colony-forming units per ml				
	Day 0	Day 2	Day 4	Day 5	Day 6
Contralateral to L-1 medium	6.4×10^3	2.5×10^3	1.3×10^2	<25	<25
" " L5178Y culture	9.1×10^3	7.5×10^3	3.1×10^6	6.3×10^6	4.5×10^6

* In U-tubes separated from contralateral side by VM Millipore filter.

TABLE II. Third Day Viable Counts of Supplemented Ultrafiltrate Cultures.

Ultrafiltrate	Dilution of ultrafiltrate with L-1 medium			
	1:5	1:10	1:20	1:40
Blank agar—Fresh	1,050,000*	1,260,000	1,400,000	1,330,000
" " —Freeze-thaw 3 ×	1,160,000	1,190,000	1,080,000	1,030,000
" " —56°C, 30 min	1,220,000	1,370,000	1,270,000	1,080,000
12-3 agar—Fresh	225,000	185,000	220,000	415,000
" " —Freeze-thaw 3 ×	160,000	165,000	250,000	325,000
" " —56°C, 30 min	195,000	230,000	205,000	435,000

* Viable cells per ml; all cultures were started with 60,000 cells/ml on day 0.

fection is not required for growth promotion.

Supplemented ultrafiltrate experiments. Since the filter barrier between the arms of the U-tube allows equally free passage of solutes in either direction, this type of experiment cannot distinguish between mechanisms involving "deficiencies" or those involving "toxins" or combinations of the two. As a first approximation to resolve which of these mechanisms is involved, sterile ultrafiltrates (*i.e.*, passed through VM Millipore filters) of infected culture lysates, and spent media of non-infected L5178Y cultures, were supplemented with 4 volumes of fresh L-1 medium, and the mixtures tested for their ability to support growth of L5178Y cells. The results of 4 such experiments are summarized in Fig. 5 and indicate that the phenomenon of lysis cannot be simply due to depletion of the medium by the mycoplasma competing for essential nutrients. On the contrary, it would seem that medium in which these mycoplasma have grown contains some toxic substance that remains when the mycoplasma are removed. This toxic substance is produced when mycoplasma growth occurs either in L5178Y cultures or on cell-free PPLO agar as shown by experiments using supplemented ultrafiltrates of agar minces extracted with medium (Table II). Growth inhibition occurred at dilutions as high as 1:40 of the active ultrafiltrate, and although much work remains to be done in characterizing the active principles, it is apparently fairly heat stable (Table II) and nondialyzable (Fig 6).

Since a variety of effects of mycoplasma on cell cultures has now been shown to be related to altered arginine metabolism(3-5), it was obviously of importance to determine whether or not our macromolecular "toxin" operated via an arginine effect. This hypothesis bore immediate fruit in experiments using sterile agar extract ultrafiltrates supplemented with fresh medium and, in addition, extra L-arginine. The standard L-1 medium is 0.71 mM with respect to L-arginine. Fig. 7 presents the results of a typical experiment in which the inhibitory property of an extract supplemented with standard medium was compared to the same extract supplemented with L-1 medium containing 2.13 mM L-arginine. With this preparation, 3 times the usual arginine completely obliterated the toxic property.

Similarly, additional L-arginine proved to be completely protective for L5178Y cells when such cultures were grown in the presence of growing lytic mycoplasma (Table III). Control cultures grew equally well over a wide range of arginine concentration (*i.e.*, up to ten times the usual level). Infected cultures supplemented with slightly less than twice the usual arginine level (*i.e.*, from 0.71 to 1.26 mM) were completely protected from lysis. This protection was unrelated to any

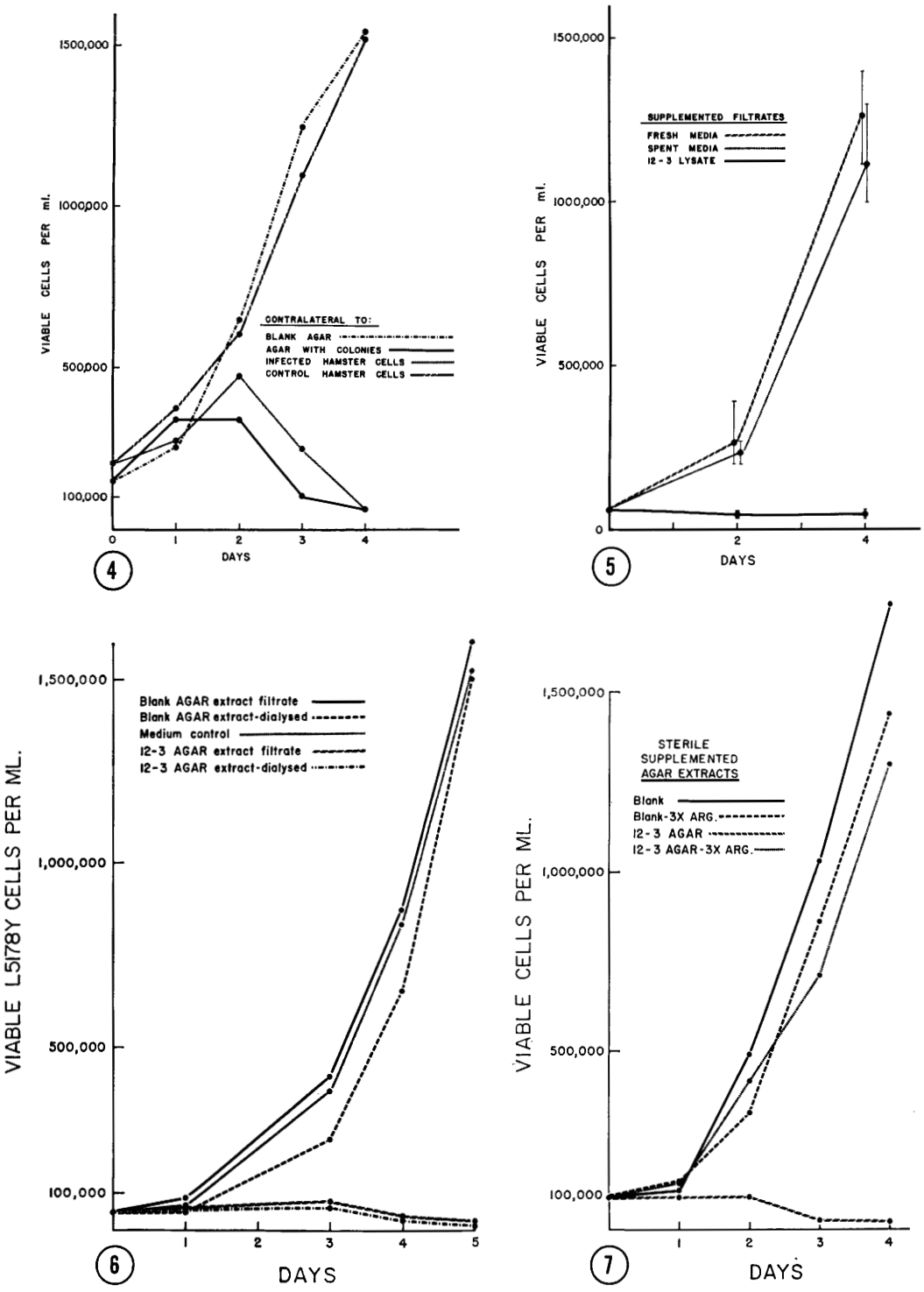

FIG. 4. Growth curves of contralateral, noninfected U-tube cultures of L5178Y cells. Ipsilateral arms were loaded with control or infected (with 12-3 strain) agar or hamster embryo cells.

TABLE III. Protection from Lysis of L5178Y Cultures Supplemented with L-Arginine-HCl.

	mM arginine	Viable cells per ml Day 1	Day 2	Day 3	Day 3 PPLO, CFU/ml
Inoculated with 12-3	36.20	185,000*	215,000	150,000	—
	18.45	265,000	450,000	680,000	1.4×10^6
	9.59	210,000	535,000	1,110,000	$.6 \times 10^6$
	5.15	320,000	675,000	1,830,000	1.8×10^6
	2.93	330,000	680,000	1,800,000	1.1×10^6
	1.82	240,000	670,000	1,930,000	1.9×10^6
	1.26	245,000	685,000	1,860,000	2.6×10^6
	.71	255,000	650,000	410,000	1.8×10^6
Control	36.20	205,000	200,000	310,000	—
	18.45	235,000	400,000	640,000	—
	9.59	315,000	540,000	1,130,000	—
	5.15	195,000	685,000	1,370,000	—
	2.93	250,000	690,000	1,850,000	—
	1.82	265,000	620,000	1,880,000	—
	1.26	270,000	630,000	1,750,000	—
	.71	245,000	680,000	1,840,000	—

* All cultures started with 100,000 cells/ml on day 0.

effect of the arginine level on the mycoplasma growth, since yields from these infected cultures were not significantly different at the various arginine levels used.

Discussion. Our results indicate that those strains of mycoplasma that we have called "lytic mycoplasma" release into the medium macromolecular substances toxic for L5178Y cells but not for many other types of tissue culture cells. Whether these substances should be considered "exotoxins" or "endotoxins" is not known, but in either case they are completely neutralizable if sufficient L-arginine is added to the medium, and are produced by these mycoplasma strains under a variety of growth conditions.

It is, of course, well known that mycoplasma as well as many other bacteria can, under some circumstances, degrade arginine to citrulline or ornithine(6,7). However, the picture is complicated by clear-cut differences in activity against L5178Y cells manifested by different mycoplasma strains. The B-7 strain, which is serologically identical to "12-3," has been identified as *M. hominis,* Type I (D. G.ff. Edward and R. H. Leach, personal communication). Three other *M. hominis* Type I strains provided by Edward (H50, H26, D419) are non-lytic. Whether or not mycoplasmal strain differences are qualitative or quantitative is not known.

In some respects, our results parallel those of Kenny and Pollack(4), who found that PPLO-contaminated cell cultures grew more slowly and with a shorter logarithmic growth phase than PPLO-free cultures, and that this effect was preventable if the arginine level of the medium was raised from 0.1 mM to 1.0 mM. Their results differ from ours in two major ways. First, they looked for, but failed to find, any residual arginine-depleting power in PPLO depleted medium sterilized with kanamycin. This is in contrast to our results with supplemented ultrafiltrates of materials in which mycoplasma had grown and which contain a non-dialyzable "toxic" material neutralizable with arginine. Second, their mycoplasma-contaminated cultures survived, although at a reduced growth rate; whereas, the murine lymphoma cultures are

FIG. 5. Growth curves of L5178Y cells in sterile ultrafiltrates diluted 1:5 with fresh L-1 medium. The spent media derived from control L5178Y cell cultures that had reached plateau counts. The data represent means and ranges of 4 similar experiments.

FIG. 6. Growth curves of L5178Y cells in sterile ultrafiltrates diluted 1:5 with fresh L-1 medium. One curve represents a 12-3 strain preparation that had been dialyzed in the cold against 200 volumes of phosphate buffered saline (containing Mg^{++} and Ca^{++}) twice for 12 hr each. The other curves are the appropriate controls.

FIG. 7. Growth curves of L5178Y cells in sterile ultrafiltrates diluted 1:5 with fresh L-1 medium or 1:5 with fresh L-1 medium containing 3 times the usual amount of L-arginine.

destroyed without any surviving cells under our conditions.

Schimke and Barile reported(8) the presence of a 3-enzyme system that degrades arginine to ornithine in PPLO-contaminated but not PPLO-free cell cultures. No association was made between the presence of this enzyme system and destruction of the cells. These workers have not reported differences between PPLO strains in this regard and have even suggested that assays of one of these enzymes, arginine deaminase, might be a suitable general screening method for PPLO detection in cell cultures. Experiments are now in progress to ascertain whether or not our lytic factor is identical to these enzymes. It nevertheless appears certain that the peculiar response of murine lymphoma cells to these phenomena is both distinctive and intriguing, and one is reminded of the antilymphoma properties that have recently been associated with guinea pig serum asparaginase(9). Our preliminary results indicate that these cells have an *in vitro* arginine requirement of about 0.15 mM, and can utilize citrulline but not ornithine in place of arginine. In this respect they are not unusual(10-12). Further work is now being done to clarify the nature of the macromolecular principle and its effect on L5178Y cells.

Summary. Evidence is presented which indicates that some mycoplasma strains which are cytocidal for murine lymphoma cells *in vitro* liberate into the medium non-dialyzable substances that are sufficient for production of the cytocidal effect. This effect is prevented if enough L-arginine is added to the medium.

Appreciation is extended to Dr. Vittorio Defendi for his active interest and advice throughout these studies.

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Received October 2, 1963. P.S.E.B.M., 1964, v115.

Enzyme Histochemistry of Rabbit Aorta, in Spontaneous Lesions and After Acute Exercise.* (28872)

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In studying the effect of exercise on enzyme content of experimental cholesterol atherosclerosis(5) and spontaneous medial degeneration of the rabbit aorta(6) it was found that there were no changes in aortic content of succinic dehydrogenase, cholinesterase, nonspecific esterase, glucose-6-phosphatase, alkaline phosphatase, cytochrome oxidase, and diphosphonucleotide diaphorase attributable to the exercise. The experiments cited above were carried out over 60-day periods. It was then postulated that observable but evanescent changes in enzyme content of the aortic wall might occur after a single intense period of exercise which were not evident in the chronic experiment. It

* This work was supported by grant from Nat. Heart Inst., Nat. Inst. Health, U.S.P.H.S.