flow through vessels capable of exchange. 5. Direct determinations of lymph flow rates from the "iliocolic" duct were found to be almost as great as flow from the thoracic duct. However, occlusion of the "iliocolic" duct diminishes thoracic duct flow by only a very small fraction. This paradox can be explained by the existence of connections between abdominal lymphatics and veins.

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Mycoplasma (PPLO) from Covertly Contaminated Tissue Cultures: Differences in Arginine Degradation Between Strains.* (29734)

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Mycoplasma isolated from covertly contaminated tissue cultures were divided into two types according to their effect on L5178Y cell cultures(1). The growth of "lytic" strains in such cultures was accomplished by a complete cytocidal response. Non-lytic strains also grew well, but this growth had no discernible effect on growth of the cells. Neither lytic nor non-lytic strains produced any evident cytopathology when inoculated into monolayer cultures of monkey kidney, HeLa, or early passage cultures of mouse, hamster, or chicken embryos. Thus, all of these strains differed from those reported by Stern *et al* which showed a pronounced cytopathic effect on various monolayer cultures (2).

The cytocidal response of L5178Y cell cultures to the "lytic" strains has been asso-

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ciated with extracellular, non-dialyzable materials that are produced by the mycoplasma whether grown in L5178Y cell cultures, in non-susceptible monolayer cultures, or even in cell-free broth or agar(3). The cytocidal response was completely abolished if sufficient L-arginine was added to the system.

The present report associates the lytic reaction with the production of arginine degrading enzymes and discusses the pitfalls of using such biochemical characteristics in detection methods for mycoplasma contamination of tissue cultures.

Materials and methods. The basic techniques for handling mycoplasma and L5178Y cell cultures have been described(1,3). Briefly, L5178Y cells are of murine lymphoma origin(4) and are carried *in vitro* as monodisperse stationary suspensions using L-1 medium. Under these conditions they attain peak cell counts between 1.5 and 2.5 million cells/ml. Since these cells do not adhere to glass or to each other, both subcultivation and serial counting of individual cultures at various times are particularly convenient. Viable counts were determined using a hemocytometer and 1:2 dilutions of resuspended cultures in 0.5% trypan blue.

Mycoplasma were grown on agar or in broth cultures using the recipes designed for cultivation of the Eaton mycoplasma(5). Titrations of mycoplasma were done by plating 0.01 ml quantities of 10-fold dilutions (in L-1 medium) of mycoplasma onto agar dishes. Results were expressed as colony-forming units (CFU)/ml. The mycoplasma strains reported here were isolated from tissue cultures without gross cytopathic changes or from virus pools derived from tissue cultures as previously reported(1).

Sterile ultrafiltrates of mycoplasma broth cultures were prepared by passage of the broth through type VM Millipore filters having an average pore size of 50 mu. In most cases, the broth was first clarified by centrifugation in a Serval centrifuge for one hour at 13,000 rpm. This was necessary to prevent rapid clogging of the filter and resultant loss of flow. The sterility of the ultrafiltrates of the lytic mycoplasma cultures was checked by serial blind passage of samples of the ultrafiltrate through L5178Y cell cultures and by plating onto agar dishes.

Chromatographic analysis of samples from experiments using C^{14} labeled arginine involved use of ion-exchange paper chromatography as described by Tuckerman(6). Samples were spotted onto WA-2 Amberlite paper in 2 μl amounts 3 times, followed by reference spots of arginine, citrulline and ornithine. The strips were developed using an aqueous acetate buffer (pH 5.2) and, after drying, were sprayed with ninhydrin reagent. Segments of the paper were eluted with 0.2 N HCl and counted using liquid scintillation methods with a Packard "Tri-Carb" automatic counter.

Arginine bioassays were done using an *Escherichia coli* mutant (strain 200) kindly provided by Dr. Werner Maas, New York University. This mutant required arginine and would not grow on either citrulline or ornithine. A standard curve was made for each experiment using turbidity as measured at 340 mu of cultures grown with variable amounts of arginine dissolved in L-1 medium. Cultures were read when the growth had reached a maximum in those grown in medium containing 1.0 mM arginine.

Results. The lytic reaction of L5178Y cell cultures refers to the sequence of events which followed the inoculation of tube cultures of these cells with certain strains of mycoplasma. The basic features of the lytic reaction, illustrated in Fig. 1, consisted of loss of cell viability and increased pH of inoculated cultures compared to control cultures. When "lytic" mycoplasma strains were inoculated into L5178Y cell cultures, this lytic reaction was accompanied and/or preceded by rapid growth of the mycoplasma. Non-lytic mycoplasma also grew rapidly in such cell cultures, but with no effect on cell growth, viability, or pH (Fig. 2).

As previously reported(3), the lytic reaction was abolished if the L-1 medium used for supporting cell growth was supplemented with additional L-arginine. This was true for all of the lytic mycoplasma strains isolated. The standard L-1 medium contained 0.71 mM arginine. As a preliminary step to further study of the lytic phenomenon, cells

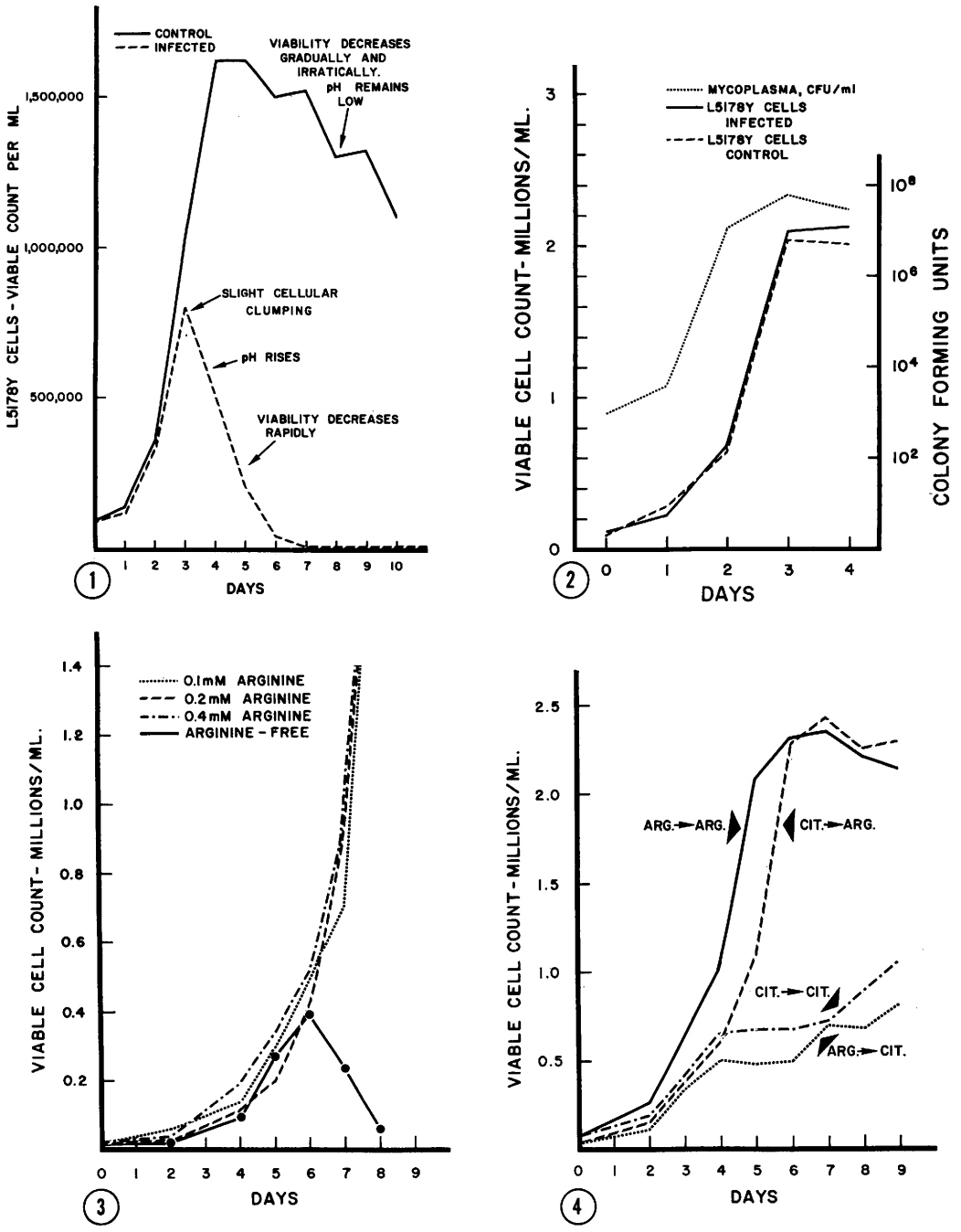


FIG. 1. Growth curves of control and infected L5178Y cell cultures illustrating the major features of the lytic reaction.

FIG. 2. Growth curves of cells and mycoplasma of an L5178Y cell culture infected with a non-lytic mycoplasma.

FIG. 3. Growth curves of L5178Y cell cultures using media containing various amounts of arginine.

FIG. 4. Growth curves of L5178Y cells with different medium histories (with respect to arginine and citrulline) when transferred to the same or different medium. Media involved were 1.5 mM with respect to either arginine or citrulline.

were grown using "arginine-free" L-1 medium to which was added various amounts of the Krebs-Henseleit intermediates, arginine, citrulline, or ornithine. Figure 3 presents growth curves of control cells in media containing between 0.0 and 0.4 mM added arginine. It is evident that these cells had an arginine requirement of the order of 0.1 mM. It was also evident that use of the "arginine-free" medium (the 5% calf serum contributed <0.05 mM arginine(7)) resulted in growth curves indistinguishable from those associated with the lytic reaction.

When cells were seeded into medium containing ornithine in place of arginine at concentrations up to 1.5 mM, no increase in growth was discernible over the small amount of residual growth associated with "arginine-free" medium. Thus it was not possible to demonstrate any utilization of ornithine by these cells.

Results with citrulline were more complicated. Initial experiments with 1.5 mM citrulline in "arginine-free" medium indicated definite growth beyond the residual growth achieved with the "arginine-free" medium. The growth rate, however, did not equal the rate achieved with medium containing as little as 0.1 mM arginine. Nevertheless, growth was progressive and a subline of cells was maintained on the 1.5 mM citrulline medium for several months.

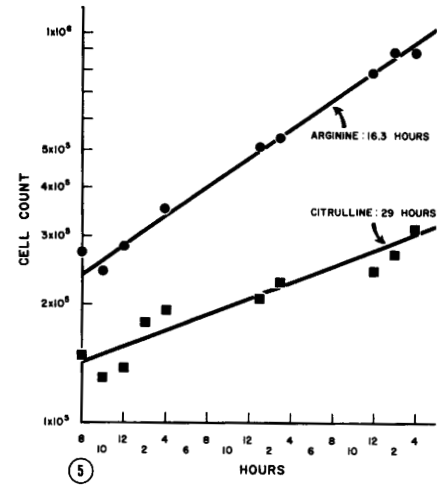
The slower growth rate of cells grown in the citrulline medium could conceivably have been due to slow formation of adaptive enzymes that synthesize arginine from citrulline. This hypothesis was tested by comparing the growth rates of cells in 4 situations: 1) cells carried in arginine medium subcultivated into fresh arginine medium; 2) cells carried in arginine medium subcultivated into citrulline medium; 3) cells carried in citrulline medium subcultivated into fresh citrulline medium; and 4) cells carried in citrulline medium subcultivated into arginine medium. In the case of adaptive enzyme lag, it would be expected that all of these cells would grow at maximal rates except for the early growth of cells transferred from arginine medium to citrulline medium. The results of this experiment are portrayed in Fig. 4. They did not support the hypothesis that the slower growth of the citrulline carried cells could be explained on the basis of adaptive enzyme lag.

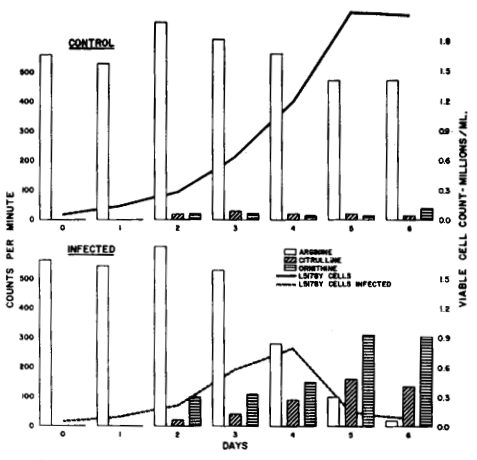
Fig. 5 gives the results of an experiment comparing the average doubling time of cells carried in the arginine medium and cells carried in the citrulline medium for 3 weeks. The experiment utilized flask cultures in "log phase" growth. Sampling was done in the warm room and counts were determined with a Coulter counter. The results indicated that even after several weeks of growth in the citrulline medium, cells continued to grow at about half the rate of cells maintained on the arginine medium.

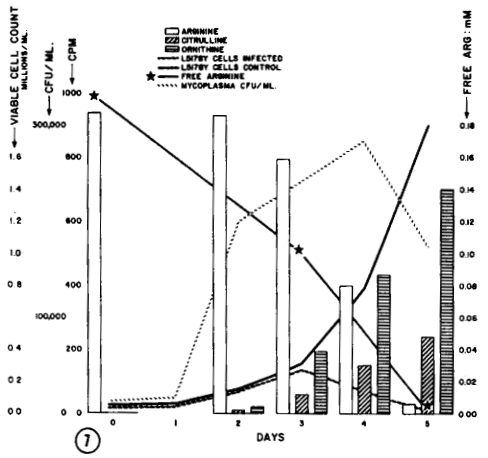
In addition to the reduced maximum growth rate of L5178Y cells carried on citrulline medium, experiments also showed that even this reduced maximum could not be achieved when citrulline was substituted for arginine on an equivalent molar basis. Whereas arginine could be reduced to less than 0.1 mM before it became limiting in the L-1 medium, citrulline concentrations of 0.8 mM or greater were required for the half growth rate.

The above results indicated that the lytic reaction of L5178Y cells could be mimicked by seeding the cells into "arginine-free" medium; that it was preventable if supplementary arginine was added; and that cell growth was not supported efficiently by citrulline and not at all by ornithine. Since it had previously been demonstrated that sterile ultrafiltrates of mycoplasma broth cultures were sufficient for production of the lytic reaction (3), the following hypothesis was proposed: the growth of lytic mycoplasma resulted in the production of extracellular materials that depleted the medium of arginine and thus caused the lysis of the L5178Y cells.

A more definitive explanation of the lytic phenomenon was then investigated using variations of the U-tube experiments described previously(3). As used in this report the two arms of each U-tube were separated by a dialysis membrane since it had been demonstrated that the active principles of the sterile ultrafiltrates were non-dialyzable(3). Both arms of the U-tubes were charged with L-1 medium containing 0.3 mM carrier argi-







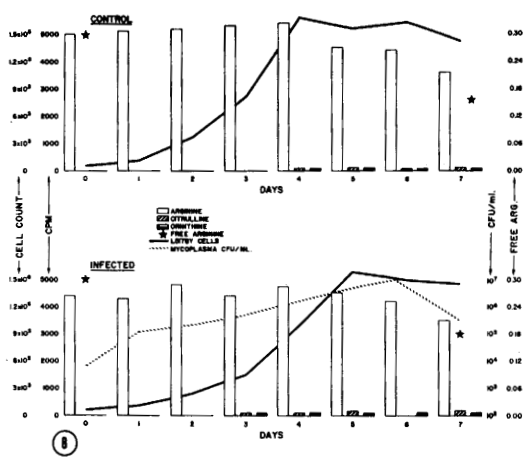


FIG. 5. Growth curves of exponentially growing flask cultures of L5178Y cells that had been carried in parallel for 3 weeks in L-1 medium containing 1.5 mM arginine for one and 1.5 mM citrulline for the other.

FIG. 6. Temporal relationship of arginine degradation, ornithine and citrulline production and cell death during the lytic reaction with "12-3" mycoplasma. Conditions of experiment described in text.

FIG. 7. Temporal relationship of arginine degradation, ornithine and citrulline production, growth curve of mycoplasma, loss of free arginine, and cell death during the lytic reaction with "LM-2" mycoplasma. Conditions of experiment described in text.

FIG. 8. Failure of a non-lytic micoplasma to cause cell death or to degrade arginine to ornithine and citrulline, or to remove free arginine, while growing to high titer in infected L5178Y cell cultures. Conditions of the experiment were similar to those used in experiments illustrated in Fig. 6 and Fig. 7.

nine and 2.5 μc arginine C^{14}. In addition, one arm of each U-tube was inoculated with L5178Y cells and sufficient calf serum to make a final concentration of 5% v/v. In addition to the cells, this arm of some U-tubes was inoculated with small amounts of either lytic or non-lytic mycoplasma. Daily samples were taken from each arm of all the U-tubes. Cell counts and mycoplasma assays (as CFU/ml) were done on samples from one arm while samples from the "medium-alone" arm were used for chromatography analysis and arginine bioassays.

Two ideas were considered in this experimental design. First, rather than study the reaction products (if any) of arginine C^{14} added to sterile ultrafiltrates of mycoplasma broth cultures, it was desired to design a sys-

tem that would allow direct correlation with the lytic reaction as it had been observed in growing L5178Y cell cultures inoculated with small amounts of mycoplasma. In this way, the information would bear some relation to the growth curves of both the cells and the mycoplasma.

Secondly, it was necessary to distinguish between two possible fates of the arginine of the medium. By use of a dialysis membrane for the U-tube medial barrier (instead of the VM Millipore® filters used previously(3)), all of the interacting principles of the lytic reaction except arginine were restricted to one arm. Therefore, by taking samples from the other arm, alternative fates of the arginine could be distinguished. For instance, the disappearance of the arginine from the "medium-alone" side might be found to be accompanied by the appearance of some reaction products. Such products would necessarily represent back diffusion from a reaction that took place in the other arm. Alternatively, the arginine might disappear without the appearance of any identifiable products, possibly implying that the arginine diffused across the membrane to be either trapped by some toxic macromolecule, or incorporated into the cells or mycoplasma.

Fig. 6 presents results of such an experiment using the 12-3 strain of lytic mycoplasma. In the uninfected U-tube no great change in arginine level occurred within the 6-day period of observation. The growth of the L5178Y cells to the plateau level in this arm was not associated with loss of much arginine. In fact, taking the CPM values literally, it was estimated that about 100 CPM out of the initial 600 CPM of arginine disappeared, which is equivalent to a reduction of arginine concentration from 0.3 mM to 0.25 mM. This is consistent with what has already been shown concerning the arginine requirement of L5178Y cells under these conditions. Furthermore, the results show that the loss of this small amount of arginine in the uninfected U-tube was not accompanied by an increase in the amounts of materials that could be identified chromatographically. It is concluded that this arginine was incorporated into the growing cells.

The results for the infected U-tube were markedly different. During the course of the 6-day period, arginine progressively diminished, and reached zero as the lytic reaction went to completion. This disappearance was accompanied, on a day by day basis, by the appearance of citrulline and ornithine. The loss of arginine was accounted for, in terms of CPM, by the appearance of citrulline and ornithine. Considering the inevitable volume variability associated with the spotting technique, and the inefficient equilibration due to the geometry of the U-tubes, this result suggested that the lytic reaction was related to the breakdown of arginine to citrulline and ornithine.

In a similar experiment, the serologically unrelated(1) lytic mycoplasma, LM-2 was used for the infected U-tube. Results for the uninfected tube were similar to the experiment above; i.e., the L5178Y cells grew to plateau levels with only minor loss of arginine and without the appearance of significant amounts of ornithine or citrulline in the medium. Results for the U-tube inoculated with LM-2 mycoplasma (Fig. 7) again demonstrated the disappearance of arginine accompanied by the appearance of equivalent amounts of citrulline and ornithine in the medium. In addition, the bioassay results independently showed that at time of completion of the lytic reaction, no free arginine was detectable. There was also a significant correlation between the timing of the lytic reaction as defined by arginine degradation and the growth curve of the mycoplasma. In previous work this correlation was found to be characteristic of the lytic reaction(1).

These results with lytic strains of mycoplasma are to be compared with results of similar experiments using a non-lytic strain (i.e., defined as a strain that had no effect on the growth of L5178Y cell cultures). Results of such an experiment are presented in Fig. 8. The data show that this non-lytic mycoplasma grew well in conjunction with the L5178Y cells but there was no significant difference between the growth curves of infected and non-infected L5178Y cell cultures. These was no significant difference in the bioassay determinations for free arginine or

any significant appearance of ornithine or citrulline. At termination of the experiment, when both cells and mycoplasma reached plateau levels, arginine concentration was not limiting.

A few experiments were also done with active sterile ultrafiltrates of lytic mycoplasma broth cultures. Such ultrafiltrates were defined as active on the basis of production of the lytic reaction when diluted 1:5 with standard L-1 medium and then inoculated with L5178Y cells. These experiments involved chromatography analysis of the reaction mixture at various times after addition of labeled arginine. Arginine was rapidly degraded by these ultrafiltrates, but all arginine degrading activity was lost if the ultrafiltrate was first heated at 95°C for 5 minutes.

The above results indicate that lytic mycoplasma are those strains of mycoplasma that actively degrade arginine. The L5178Y cell culture system, therefore, can be used as an indicator system for detection of such strains. Furthermore, it is evident that covertly contaminated tissue cultures may contain either or both types of mycoplasma(1). The general experience in this laboratory has been that the non-arginine-degraders have been more frequently isolated from tissue cultures than the strains that are active in this regard(1).

Discussion. Most commonly, when tissue cultures are found to be contaminated by mycoplasma, this contamination is not accompanied by overt turbidity, cytopathology, or reduced growth rate(8,9). The tissue culture sources of the mycoplasma strains studied here were no exception to this rule. Except for the response of L5178Y cell cultures, lytic and non-lytic strains could not be distinguished either in terms of the appearance of the cultures from which they were isolated, or in terms of the response following inoculation of these strains into a variety of monolayer cultures(1). It is now clear that the lytic strains produce substantial amounts, under all known conditions of growth, of arginine degrading enzymes, and that non-lytic strains do not. It seems certain that the enzymes involved include an arginine deaminase and an ornithine transcarbamylase as described by Smith(10) and Schimke and Barile(11). It is also apparent that L5178Y cell cultures are peculiarly sensitive to the presence of these enzymes under the *in vitro* growth conditions used.

The reasons for this sensitivity of L5178Y cell cultures is not completely understood, though some hypotheses can be advanced. These cells do not readily utilize citrulline in place of arginine as evidenced by the effect of citrulline substituted media on average doubling time. This effect was evident even when the citrulline concentration was raised to 10 times the molar equivalent of the minimum arginine requirement. This finding differs from that reported for many cell lines (12). Utilization of ornithine in place of arginine has not been reported for any mammalian cell culture (see, however, 13). It may also be speculated that monolayer cultures are protected from a special cytopathological process associated with limiting arginine by the occurrence of confluency inhibition of division during the latter part of the subdivision cycle. If this is true, it might imply that arginine deprivation of rapidly growing L5178Y cells results in something analogous to the unbalanced growth that has been detected in nutritionally deprived bacteria(14).

Regardless of the explanation of this characteristic of L5178Y cell cultures, it seems evident that such cultures can be used to detect contamination of tissue cultures by arginine degrading mycoplasma. It also has been directly demonstrated that some tissue cultures are contaminated with mycoplasma that do not degrade arginine under tissue culture conditions. Consequently, it must be concluded that use of an assay for citrulline production as a general method of detecting mycoplasma contamination of tissue cultures (15,16) could leave many mycoplasma undetected.

It is not surprising that such a test might prove inadequate. An ever increasing body of evidence points to both considerable heterogeneity among strains (reviewed recently by Smith(17)), and to considerable complexity of the biochemical machinery of this group of organisms. Recent evidence for

presence of bacteria-like ribosomes and nuclear bodies(18), extensive pathways such as Emden - Meyerhoff(19), hexose monophosphate shunt(20), and a typical amino acid incorporation system(21) are indicative of their complexity. Their heterogeneity can be illustrated in terms of sterol requirements and carotenoid pigment production(22), carbohydrate fermentative abilities(23), hemolysin production(24), etc., as well as the strain differences reported here. Taken as a group, with the defining exception of an inability to synthesize cell wall mucopeptides and various consequences thereof, mycoplasma seem sufficiently similar to bacteria to consider them, operationally if not in fact, as cell wall deletion (i.e., non-reverting) mutants of as yet unidentified bacteria.

This viewpoint includes recognition of the possibility that the mycoplasma group may include derivative organisms from a wide spectrum of bacteria genera. When it is also considered that tissue culture contamination is evidently of external origin(25), it might be expected that these contaminants would represent a spectrum of strains whose natural ecology overlaps the locale of the people, animals, and by-products of laboratories using tissue cultures. Hence, a suitable detection method for routine use must be both highly sensitive to small numbers of organisms and be sufficiently non-restrictive to allow detection of all common variants. The citrulline production test is deficient in both of these requirements.

A more general objection must be registered in regard to the types of evidence frequently used as criteria of reliability of particular detection methods(29,27). This type of evidence says, in effect, that X number of cell lines from Y laboratories were examined by two methods, A and B; since the same cultures were found to be positive using newer method B as older method A, therefore B can be used for routine purposes in place of A. This is an unreliable method of reasoning because 1) strain classification of mycoplasma is still very primitive (see 17), preventing any valid impression of the range of types in a particular collection of tissue culture isolates, and 2) considering the "epidemiology" of tissue culture contamination (26), the degree of independence of the various positive cultures from the collection of tissue cultures is indeterminant. In the extreme case, all of the strains detected in the above example might be a single variety that can be detected equally well by the two methods. Nevertheless, method A might be intrinsically more versatile.

In consideration of the above, it seems likely that, just as the citrulline production test appears to be insufficient, the more recent thymidine cleavage test of Horoszewicz and Grace(27) will also prove to be an unwise utilization of interesting biochemical results. These attempts seem entirely analogous to attempting to use a single marker, such as, for example, lactose fermentation, to test for the presence of bacteria in general.

Summary. The lytic reaction of L5178Y cell cultures to certain mycoplasma strains is associated with the release into the medium of arginine degrading enzymes. Mycoplasma isolated from covertly contaminated tissue cultures include strains that degrade arginine and strains that do not. These two types of strains can be distinguished by inoculation into L5178Y cell cultures. The use of biochemical markers, such as arginine degradation, for routine detection of mycoplasma is critically discussed.

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A Method for Eradication of *Mycoplasma* Infections in Cell Cultures (29735)

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Investigators using cell cultures have become increasingly aware of the problems introduced by parasites such as *Mycoplasma*.

In the past few years many have attempted to eliminate *Mycoplasma* in tissue cultures, using different methods and with varying degrees of success. Heat treatment (Hayflick (7)), use of specific antiserum (Pollock and Kenny(2)) or antibiotic treatment (Hearn, Officer, Elsner, and Brown(3); Pollock, Kenny and Syverton(4); Koehler(5)) have all been suggested. In our hands these methods have not been consistent, in that they may have been successful only in particular cell lines and with particular strains of *Mycoplasma* contaminants, or on account of more sensitive methods (Hayflick(1)) used in our laboratory to isolate aerophilic and microaerophilic *Mycoplasma* (Table I). As the morphology of isolates suggested that a variety of *Mycoplasma* was infecting the screened cell lines, the preparation of polyvalent antisera was not considered practical, and it was decided instead to explore fully the usefulness of antibiotic treatment. Simple antibiotic treatment, even at near cytotoxic doses and for periods of up to 5 months, proved only capable of depressing the severity of infection, approximately from 100 or more to 10 or less colonies per million cells, without effecting sterilization (Table I). One obvious difficulty was the fact that *Mycoplasma* infection can be and usually is an intracellular infection (Edwards and Fogh (6)); Hayflick and Stinebring(7); Carski and Shepard(8)). In consequence, antibiotics or specific antisera may have eliminated the *Mycoplasma* present in culture fluids without affecting those nested inside cells. A possible approach was to increase the permeability of the cells and, of the several possible methods, a simple hypotonic treatment was selected.

Materials and methods. Antibiotic-Hypotonic treatment. Previous studies (Blyth(9); Hearn, Officer, Elsner, and Brown(3); Fogh and Hacker(10); Pollock, Kenny and Syverton(4); Koehler(5)) indicate that *Mycoplasma* are highly sensitive to aureomycin,