present in plasma at various concentrations ranging from 0.001 to 0.01 mM where apparently they are largely protein bound(20), but the concentrations at sites of biological mineralization are unknown. Zinc was the only element used that completely inhibited mineralization at concentrations detected in plasma, *i.e.*, 0.015 mM(8), but the data reported indicate that the inhibiting metallic ions may act additively in preventing mineralization. Therefore, total concentration of the various inhibiting ions may be more pertinent than that of a single inhibitory element to problems of bone mineral deposition. Whether aberrations in transport or concentration of these elements in any way contribute to clinical disorders of mineralization requires further study.

Conclusion. The inhibitory effect of various metals on mineralization *in vitro* and on apatite crystal formation *per se* have been determined. Zinc, cadmium, manganese and cobalt inhibited mineralization of rachitic cartilage matrix when present at concentrations of 0.10 mM or less. Cobalt was unique in preventing apatite crystal formation at concentrations inhibiting mineralization of matrix. These observations indicate that the possible inhibitory effect of certain metals must be considered in any study of mineralization involving use of biological fluids.

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Experimental Infection of Tissue Cultures with Certain Mycoplasma (PPLO).* (28127)

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For some time virologists have been aware of complications which might arise as a consequence of tissue culture contamination with pleuropneumonia-like organisms (PPLO). The problem has become increasingly important since so many host-cell systems can now be grown in continuous culture as well as by primary explant technics(1,2). Soon after

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observing incidental PPLO contamination and persistence in established cell lines Hayflick and Stinebring(3) were able to initiate experimental PPLO infections in the embryonated egg and in various primary cell cultures, where growth was observed both intracellularly and extracellularly. Recent metabolic studies(4) indicated that the pattern of amino acid metabolism in tissue culture was altered when cell lines were infected with large PPLO populations; however when systems were infected with numbers of PPLO usually found as contaminants in tissue cultures, no change in the amino acid metabolic pattern was observed. For the most part, media routinely used to cultivate mammalian cells did not permit replication of the usual PPLO contaminants; however when HeLa cells were present, PPLO multiplied(5). Nelson(6) demonstrated that HeLa cells infected with murine PPLO showed marked cytopathogenic alteration of the monolayer, following successive renewal of growth fluids over prolonged testing intervals. The present study was undertaken to detect metabolic alterations following infection of cultured human cells with certain pathogenic and saprophytic PPLO, particularly those changes which might supplement cellular energy-yielding mechanisms as a result of PPLO influence. Preliminary studies indicated that of the 5 cell lines tested (FL, DMB, D. Hov., HeLa and Chang conjunctival) the FL cells were most susceptible to the various stock PPLO we wished to use for such investigations.

Materials and methods. Dr. D. G. Edward, Wellcome Research Laboratories, England, kindly supplied cultures of *Mycoplasma laidlawii, M. hominis* and *M. agalactiae*; Dr. H. E. Adler, University of California, Davis, provided a strain of *M. gallisepticum.* The PPLO were maintained in heart infusion broth (Difco), supplemented with 10% sterile horse serum which was purchased from Microbiological Associates and checked for absence of PPLO before it was included in culture media. Two media were used to determine viable numbers; the solid medium contained PPLO agar (Difco) and 10% horse serum while the liquid medium consisted of heart infusion broth, 10% horse serum, 0.8% glucose and phenol red indicator. Decimal dilutions of inocula were plated on solid media and cultivated in liquid broth in order to determine approximate viable cell numbers. The FL strain of human amnion cells obtained originally from Microbiological Associates had been transferred 24 times in this laboratory; the last 5 passages were shown to be free of endogenous PPLO following treatment with aureomycin in concentrations of 2.5 μg/ml. Eagle's medium containing 10% calf serum served as culture fluid for amnion cells.

Inoculum preparation. PPLO were grown 48 hours at 37°C in heart infusion broth containing 10% horse serum. Cultures were then centrifuged at 1000 $\times$ *g* for 1 hour and the PPLO resuspended in phosphate buffered saline, pH 7. Each tissue culture bottle was inoculated with 2.5 ml of suspension containing 10^8 viable particles per ml.

Phagocytosis mixture. The phagocytosis mixture(7) consisted of 3.5 ml saline buffered with phosphate (0.1 M, pH 7), 700 μmoles glucose, 350 μmoles Mg^{++}, 350 μmoles Ca^{++}, 1.75 ml horse serum and an additional 2.5 ml buffered saline containing 2.5 $\times$ 10^8 PPLO.

Infection of monolayer. Milk dilution bottles (200 ml) were seeded with 10^6 FL cells suspended in 10 ml Eagle's medium containing 10% calf serum and cultures were incubated 48 hours at 37°C. Although a confluent layer had not developed, cells were sufficiently stretched out to allow maximum cell surface exposure for phagocytosis. Before inoculation the cell sheets were washed once with buffered saline, after which the phagocytosis mixture was introduced. Preparations were held 90 minutes at 30°C; supernatant fluids were discarded and monolayers were washed again with buffered saline to eliminate superficial organisms. Eagle's medium was added and cultures were then incubated at 37°C until PPLO growth could be detected.

Fermentation studies. Because of the complex medium required for growth of *M. gallisepticum* it was necessary to incorporate a radioactive tracer in growth fluids in order to identify the products of glucose or lactate metabolism. Radioactivity recoveries were calculated from culture samples when *M. gal-*

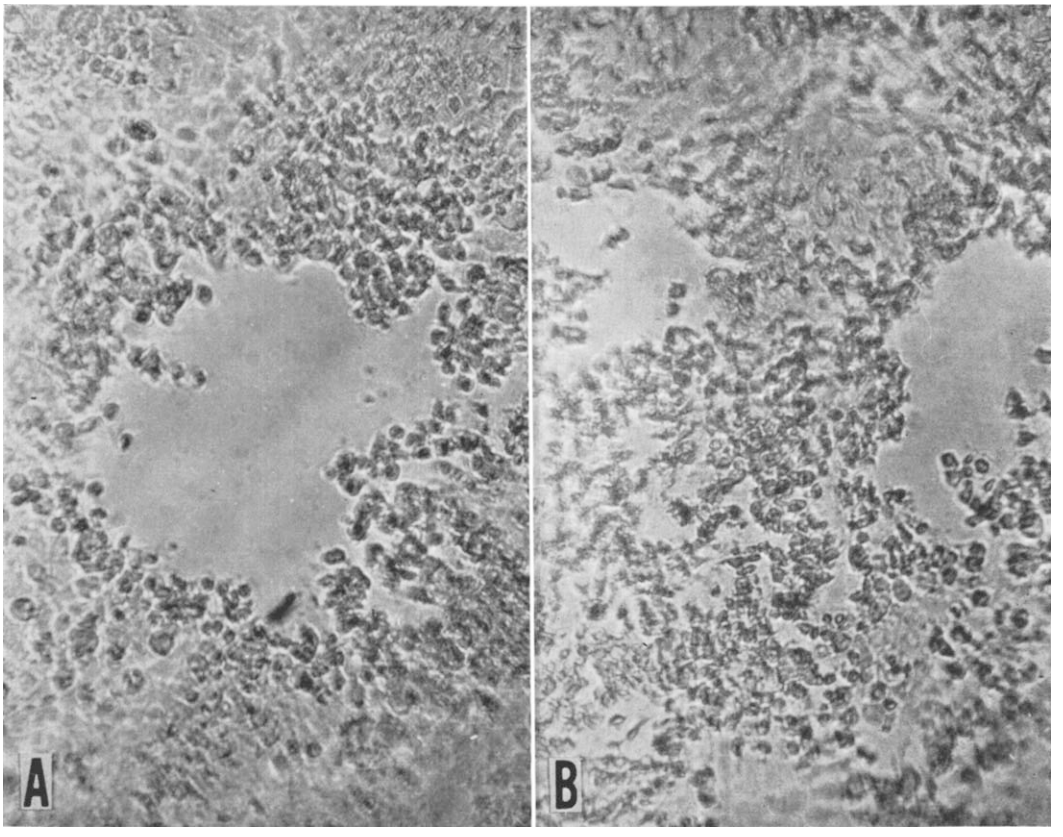

FIG. 1. Cytopathogenic effect observed in FL cells infected with *M. gallisepticum* 3 wk after infection. Magnification 75 ×.

lisepticum was grown in tryptic digest broth (Difco) containing 10% horse serum and 2 μc uniformly C^{14}-labeled glucose or lactate-2-C^{14}. Cultures were incubated at 37°C for 24 and 48 hours. After appropriate incubation intervals, acids in fermentation liquors were separated by celite column chromatography(8); then aliquots from each fraction were plated and radioactivity determined in an ultra-thin window nuclear gas flow counter. Glucose was measured by the glucose oxidase test using reagents of the Worthington Laboratories(9).

Results. Following inoculation of amnion cells, the saprophytic strain *M. laidlawii* A was found to disappear from cultures after 48 hours' incubation. FL cells infected with *M. agalactiae* and *M. hominis* were carried a total of 3 weeks, during which time one-fifth of the cells were transferred to new bottles at 6-day intervals. After 3 weeks' cultivation under these conditions PPLO could be demonstrated by plating supernates and washed cells on solid medium. No cytopathogenic effect was observed in these cultures during the test intervals when cultures were transferred every 6 days. Cell cultures infected with *M. gallisepticum* were treated similarly throughout the experiment and after 3 weeks were found to show signs of cytopathogenic effect as described by Nelson(6) in his studies with murine PPLO-infected HeLa cells. The cell sheet showed patches where cells had rounded up and detached from the glass surfaces (Fig. 1).

M. gallisepticum is an organism capable of dissimilating glucose with the production of acid. To observe the effect of tissue culture infection by these organisms, in terms of accumulation of products from the fermentation of glucose, supernatant growth fluids of infected and non-infected tissue cultures were

TABLE I. Fermentation Products Following Growth of *M. gallisepticum* in FL Tissue Cultures.

	Acetate	Pyruvate	Lactate
	μmoles/10 ml		
Medium alone	16.5	14.0	25.0
Control 1	15.4	19.8	79.2
" 2	11.0	19.0	85.0
" 3	13.0	13.2	78.0
Infected 1	27.5	23.0	82.5
" 2	21.0	17.6	81.4
" 3	21.0	21.0	78.0

Cells infected as described in text were fed at 48- and 96-hr intervals after inoculation. Growth fluids used for the above tests were collected from cultures 24 hr after the last feeding. Organic acids separated by celite column chromatography are reported as μmoles products per 10 ml test sample. Cultures were grown in Eagle's medium containing 10% calf serum.

studied. Cell monolayers were inoculated as described in the *Methods* section and fed twice at 48-hour intervals; the culture fluids obtained for study were collected during the interval of 96 to 120 hours following infection. When organic acids were separated by celite column chromatography from 1 ml culture fluid samples it could be observed that the acetate concentration had increased in infected systems but this increase could not be detected in media controls or in uninfected tissue culture preparations (Table I).

In fermentation studies with *M. gallisepticum,* carried out to identify the products of glucose metabolism, uniformly labeled C^{14}-glucose was dissimilated to acetate, lactate and CO_2 with a 96% radioactivity recovered in the products. These data are reported in Table II. Similar experiments using lactate-2-C^{14} as substrate were terminated after 24- and 48-hour incubation intervals (Table III). In these studies some lactate was used as acetate was formed, although only 75% of the total radioactivity was recovered. It is assumed that the 25% deficit may possibly be accounted for by PPLO assimilatory processes.

TABLE II. Glucose Dissimilation by *M. gallisepticum* in Absence of FL Cells, but When Grown in a Complex Medium Containing C^{14} Labeled Glucose.

	cts/min added substrate	cts/min products
Glucose	796,000	
CO_2		10,400
Acetate		157,500
Lactate		597,500
Total	796,000	765,400
Recovery	96%	

Organisms were grown in 14 ml tryptic digest broth containing 10% horse serum and 2 μc uniformly labeled C^{14}-glucose. Medium was inoculated with 1 ml 48-hr PPLO broth culture and incubated 24 hr.

Discussion. Chemical assay of culture fluids following growth of non-infected and infected FL cells with *M. gallisepticum* showed an increased acetate concentration in PPLO-infected systems. In studying the dissimilation of glucose by *M. gallisepticum* alone, fermentation balances based on the recovery of radioactivity in products following growth of an agent in a system containing labeled substrate proved useful in these studies particularly since complex nutrients were required for growth of this PPLO(10). These studies indicated that *M. gallisepticum* dissimilates glucose with the production of lactate, acetate and CO_2, but when glucose is substituted by lactate, the latter disappears and acetate is formed. These data suggest that the increased acetate concentration found in tissue cultures infected with *M. gallisepticum* is probably due to metabolic activities of the parasitic PPLO whereby the agent may dissimilate some of the lactate allowing acetate to accumulate as a product. In addition part of the acetate may be assimilated since 25% of the radioactivity was not recovered in the products.

TABLE III. Lactate-2-C^{14} Dissimilation by *M. gallisepticum.*

	cts/min added substrate	24 hr cts/min products	48 hr cts/min products
Lactate added	635,000		
CO_2		0	0
Acetate		89,750	177,750
Pyruvate		0	0
Lactate remaining		374,750	312,000
Total	635,000	464,500	489,750
Recovery		73%	77%

M. gallisepticum was grown in 14 ml tryptic digest broth containing 10% horse serum and 1 mc lactate-2-C^{14}. Medium was inoculated with 1 ml of a 48-hr PPLO broth culture. Samples for testing were removed after 24- and 48-hr incubation intervals.

These observations suggest the possibility of dividing the *Mycoplasma* into 3 groups according to their effect on mammalian cells growing in tissue culture. The rather general working classification outlined below may prove useful and convenient in distinguishing saprophytic from pathogenic PPLO. Perhaps the first large division might include saprophytic Mycoplasma such as *M. laidlawii* which shows little if any growth or multiplication in tissue cultures. PPLO considered to be primarily endogenous tissue culture contaminants, in that they persist in small numbers and establish an ecological equilibrium with the host, may comprise another large group. The third division could be composed of those PPLO thought to be potentially pathogenic for cell lines when maintained 2 to 3 weeks in tissue culture, particularly when PPLO metabolic processes supplement or alter host cell metabolism. The reported data suggest that the strain of *M. gallisepticum* studied, when growing in FL cells is a rather typical agent according to the third suggested PPLO grouping.

Summary. Infection of FL tissue culture cells with *M. gallisepticum, M. agalactiae* and *M. hominis* was achieved, while the saprophytic PPLO *M. laidlawii* A could not be detected in such cells when cultivated for intervals longer than 2 days following infection. Parasitic PPLO remained in cell cultures for periods up to 3 weeks. After prolonged incubation, cytopathogenic effect could be demonstrated in cultures infected with *M. gallisepticum.* In addition, tissue cultures infected with *M. gallisepticum* showed an accumulation of acetate in growth fluids. The latter observation, supported by appropriate isotope data, strongly suggests that acetate appears as a product of lactate dissimilation by this PPLO.

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Evidence of Monoamine Oxidase Inhibition by Myristicin and Nutmeg. (28128)

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Nutmeg, the seed of *Myristica fragrans,* contains a terpene-like compound, myristicin, which for years has been identified as the principal active ingredient. This early observation of Sir Henry Dale has been recently confirmed using both animal and human tests (1). Nutmeg intoxication produces an unusual syndrome in man involving, first, stimulation and a feeling of euphoria. This may extend into an acute toxic psychosis with an overdose and may be followed by a depressed state during recovery.

There is a degree of structural resemblance between the chemical formula for myristicin and those of certain sympathomimetic amines. This is especially true when the methylenic group of myristicin is considered as isosteric with an amino group. This analogy is shown in Fig. 1. This similarity coupled with the stimulating action of nutmeg prompted a preliminary evaluation of myristicin and crude ground nutmeg for evidence of central monoamine oxidase inhibition.

Materials and methods. Ground East In-