more potent than the parent in the Clauberg test, was less potent as an estrone antagonist; thus the ratio was grossly reduced. Incorporation of a double bond at the 9-10 position of 17α-chloroethynyl-13β-ethyl-17-hydroxygon-4-en-3-one resulted in decreases of both activities; however, since the decrease in progestational effect was greater, the ratio was considerably increased. 17α-Chloroethynyl-13β-ethyl-17-hydroxygon-4,9-dien-3-one was less potent in the Clauberg test, but more potent in the anti-estrogenic assay than the lower homologue. Thus the ratio of potencies was decreased.

13β-Ethyl-17-hydroxy-17α-methylgon-4-en-3-one was more anti-estrogenic and less progestational than its lower homologue. 17α-Ethyl-17-hydroxy-13β-methylgon-4-en-3-one (norethandrolone) was less potent in both assays than its 18-homologue (norbolethone), which because of a greater anti-estrogenic potency had a higher ratio. Incorporation of a double bond in the B-ring of norbolethone to form 13β,17α-diethyl-17-hydroxygon-4,9-dien-3-one did not alter progestational potency, whereas the anti-estrogenic potency and the ratio were decreased.

These data fail to indicate any direct correlation of progestational and anti-estrogenic effects. Although both these biological activities are often present in a single molecule, they do not appear to be necessary correlates.

Summary. The progestational (Clauberg) and anti-estrogenic (mouse vaginal smear) potencies of various Δ^4-3-oxosteroids are compared. The ratio anti-estrogenic potency/progestational potency varies from 0.1 to 85, indicating that there is no necessary correlation between these parameters of steroid action.

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Reversible Inhibition of Mitosis in Lymphocyte Cultures by Non-Viable Mycoplasma.* (31605)

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The observation by Nowell(1) that phytohemagglutin (PHA) increased mitosis in lymphocyte cultures has resulted in the application of this technique to many fields of research. Its application in immunology was established when tuberculin purified protein derivative(2) was the first of many antigens (3,4,5) used to stimulate transformation in lymphocytes from sensitized individuals. A logical extension of this basic immunologic phenomenon leads to the idea of stimulating the lymphocytes from rheumatoid arthritis patients with a suspected etiologic agent and of comparing the response to that of lymphocytes from normal controls.

Recently there have been reports of the isolation of mycoplasma (PPLO) from the synovial fluids of patients with rheumatoid arthritis and other diseases(6,7). However, confirmatory evidence is lacking on the etiological role of these organisms. An attempt was made to test the hypothesis that

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PPLO might stimulate lymphocytes from some patients with rheumatoid arthritis but, thus far, the results have been disappointing. When leukocytes from patients with inflammatory arthritis were challenged with PPLO cultured from their cervical os or prostatic secretion, the lymphocytes showed no consistent evidence of increased mitosis or enlargement or other evidence of stimulation, even though the serum from these patients contained PPLO antibodies by the PPLO-latex agglutination test(8). During this attempt the impression was gained that PPLO may be inhibiting rather than stimulating mitosis. This report presents evidence that non-viable PPLO inhibit mitosis in lymphocyte cultures and that this inhibition is reversible.

Materials and methods. The basic method of leukocyte culture as described by Moorhead *et al*(9) was followed with minimal modification. PHA (Difco) was the mitogenic agent. Penicillin and streptomycin were added to the cultures.

The PPLO were isolated and grown in guantity by methods described previously(10) and identified as *Mycoplasma hominis* type I by the growth inhibition test(11) and the PPLO-latex agglutination test(8). The suspensions of PPLO in broth were divided into convenient 0.6 ml portions, frozen and maintained in the frozen state until used. The inoculum was 0.05 ml of broth suspension containing PPLO in serial dilution (see *Results* re concentration) while control cultures were inoculated with the same amount of broth without PPLO. All cultures were terminated 4 days after planting and colchicine was introduced 4 hours before harvesting. The cells were stained either with Giemsa's stain or acetic orcein and the number of mitotic figures per 1,000 cells (mitotic index) was determined in a single blind manner.

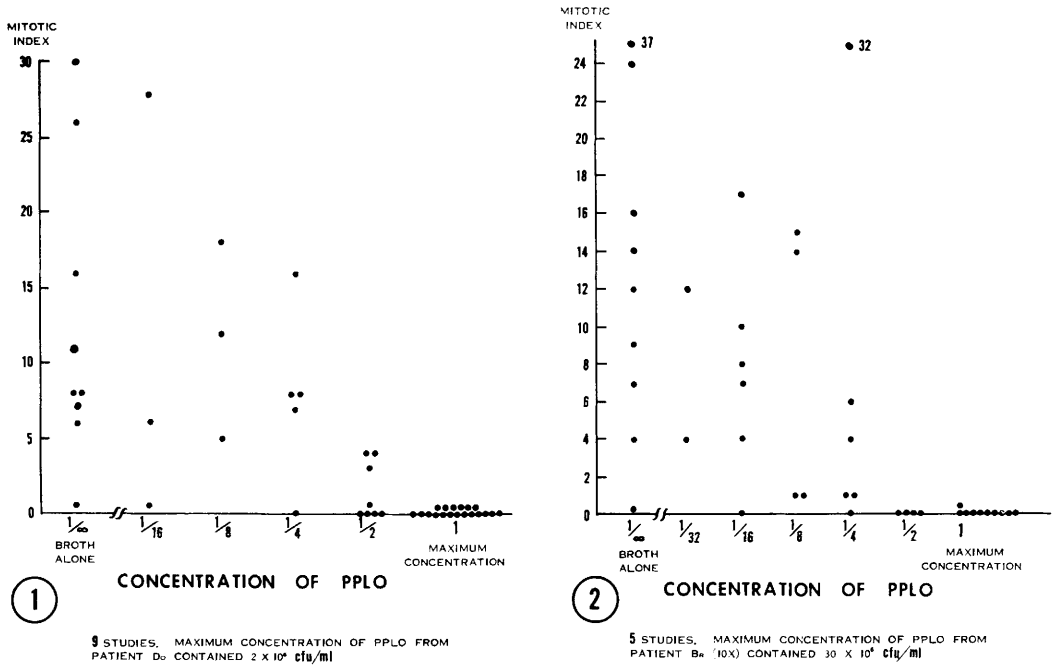
Results. Demonstration of inhibition of mitosis by PPLO. PPLO from patient Do was a reliable inhibitor of mitosis when the broth culture was used undiluted in a concentration of 2×10^6 colony forming units (CFU) per ml. Graph I, in which the mitotic index is plotted against the concentration of PPLO, is a composite of all data with this suspension. Nine control cultures (infinite dilution) yielded mitotic indices ranging between <1 and 30. At 1/4 to 1/16 of the maximum concentration 9 of the cultures gave indices ranging between 5 and 30 while 2 cultures failed to show mitosis. By contrast, when the organisms were used undiluted, there was practically a complete absence of mitosis. Thirteen cultures showed no mitotic figures and 6 cultures yielded a mitotic index of <1. When diluted with an equal quantity of broth the results suggested a transition point since an occasional culture yielded a mitotic index of 3 and 4.

The undiluted suspension of PPLO from patient Br also showed inhibition of mitosis in the only 2 cultures to which it was added. In order to obtain a more concentrated suspension of PPLO, a broth culture was centrifuged at 17,000 rpm for 1/2 hour and the sediment resuspended in broth to 1/10 of the original volume. It was designated as PPLO (Br 10×) and contained 30×10^6 CFU/ml. It was stored in the frozen state, thawed before each study and then refrozen.

The supernate from the above centrifuged broth cultures of PPLO contained relatively few organisms and did not inhibit mitosis. This ruled out a soluble extracellular substance as the inhibitor.

Graph II shows the results of all studies using PPLO (Br 10×). Again the wide range of mitotic indices (0-37) for the control cultures containing broth alone is noted. A similar scatter for cultures with PPLO (Br 10×) in a dilution of 1/4 or greater was obtained. By contrast the inhibition demonstrated by the undiluted suspension and the 1:2 dilution is complete and consistent.

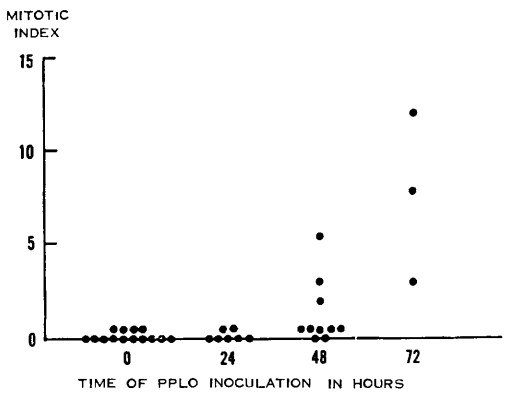
In order to delineate this inhibition phenomenon, inoculations with PPLO suspensions were made at times other than the time of planting ($t = 0$). Graph III shows the mitotic index plotted against the time of PPLO inoculation and even though the mitotic index is low, it is apparent that inhibition is essentially complete within the first 24 hours and escape from inhibition occurs thereafter. The 29 cultures with a mitotic index of either 0 or <1 plotted at the point of maximum concentration in Graphs I and II include all



Graph I and Graph II.

cultures inoculated with PHA and PPLO within 24 hours of planting. Inoculations made after 24 hours as shown in Graph III are not included in Graphs I and II.

Reversal of PPLO inhibtion of mitosis. To show that mitogenesis by PHA was inhibited by PPLO and that the failure to produce mitosis was not merely the effect of a toxic substance, evidence of lymphocyte viability was sought. Preliminary results with the Trypan Blue dye exclusion test, and the demonstration that cultures with PPLO survived as long as cultures without PPLO were consistent with the interpretation that the lymphocytes were viable and intact. Additional and more convincing evidence of viability was obtained by demonstrating reversal of inhibition and the production of mitosis.



Graph III.

In Graph IV are shown the results of 26 cultures in which the attempt was made to reverse mitotic inhibition by removing the PPLO from the culture. The removal was effectuated by draining off the medium above the cells and replacing all of the ingredients, including additional PHA. The cultures were terminated as usual after a total of 4 days. Twelve cultures had PPLO for the first 24 hours and after removal mitosis was obtained, as shown in the first column. The last column shows that even 48 hours of inhibition did not prevent the emergence of mitosis after the removal of PPLO. The second column illustrates that the mitotic process can be started for 24 hours, inhibited for the next 24 hours and the inhibition reversed at 48 hours to yield a mitotic index as high as 23.

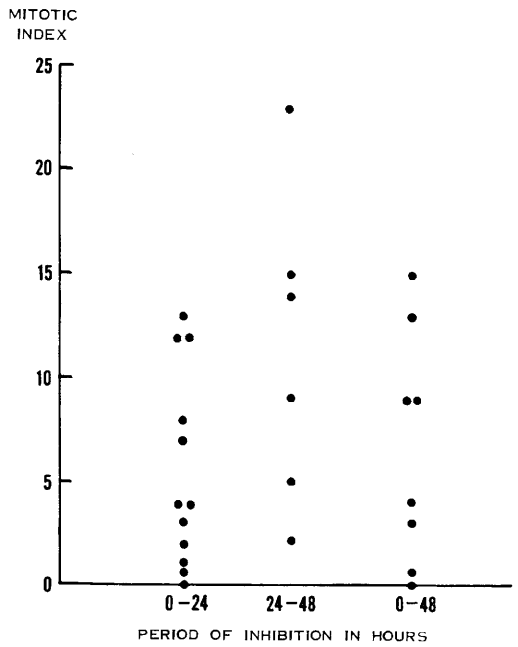
Reversal of inhibition of mitosis was demonstrated in another experiment using PPLO which had been concentrated to 100 times what they were in the original broth culture

CHART I

Row	Time in hours 0	24	48	72	96	Mitotic index Study 1	Study 2
1	PHA	Broth		Terminate		22	39
2	"	Broth			Terminate	77	39
3	"	PPLO (Br 100×) 1/32		"		0	<1
4	"	"			"	0	<1
5	"	"	Change medium, add PHA	"		8	27
6	"	"	"	"		15	33
7	"	"	Change medium, no PHA		"	20	41
8	"	"	"		"	18	54

(Br 100×). The micromethod of Hungerford (12) for culturing lymphocytes from whole blood was used. An inoculum of 0.05 ml of the PPLO (Br 100×) suspension inhibited mitosis in a dilution of 1/64. The protocol and results are summarized in Chart I.

Rows 1 and 2 are control cultures without inhibitor which yielded high mitotic indices. Rows 3 and 4 show the complete inhibition obtained with a 1/32 dilution of PPLO (Br 100×) added after 24 hours. Rows 5 and 6 show that removal of PPLO and additional PHA for 24 hours (rather than 48 hours as previously performed) will also yield significant numbers of mitotic figures. Finally, rows 7 and 8 demonstrate reversal of inhibition without adding additional PHA at the time of changing the medium.



Graph IV.

Discussion. If PPLO were mitogenically inactive or a stimulant, one would expect the same or a higher mitotic index when they are added simultaneously with PHA. Graphs I and II clearly demonstrate that sufficiently concentrated *Mycoplasma hominis* type I will inhibit mitogenesis normally initiated by PHA. It is noted that the mitotic indices of the control cultures were less than those usually obtained by other workers. This difference is due to the conditions under which the experiment was performed. However, the consistent and almost complete inhibition of mitosis in cultures with concentrated suspensions of PPLO is in marked contrast to the range of mitotic indices of cultures with diluted PPLO inocula. A total of 22 cultures failed to show even one mitotic figure and 7 cultures showed only an occasional mitotic figure (mitotic index <1).

To differentiate between toxicity and mitotic inhibition is neither obvious nor simple. An excellent test of mitotic inhibition as opposed to toxicity is the reversal of inhibition and the production of mitosis. Graph IV shows that even 48 hours of inhibition by PPLO (column 3) could be reversed by removal of PPLO. The results shown in the second column of Graph IV suggests that this technique may offer itself as a useful research tool. Mitosis can be initiated by PHA, stop-

ped with PPLO, and restarted by removal of the PPLO. A study of cellular dynamics and metabolism during this period of inhibition should be rewarding. Graph III clearly shows that PPLO inhibition is less effective after 48 hours, and it is noteworthy that this corresponds fairly closely to the period of time required for DNA replication(13).

One might criticize the studies shown in Graph IV on the grounds that "true" reversal would demand the completion of mitosis without additional PHA after removing the inhibitor. One could argue, for example, that the lymphocytes stimulated in the first 24 hours were vulnerable to the cidal action of PPLO during the second 24 hours, and that a new crop of lymphocytes was stimulated by the additional PHA after removing the PPLO "toxin." The studies shown in Chart I were designed to evaluate this argument in two ways. If a new batch of lymphocytes had been stimulated by PHA added at 48 hours (rows 5 and 6) then no mitosis would have been obtained in the following 24 hours. Rows 5 and 6 illustrate the continuation of the mitotic process initiated at time t = 0 and inhibited at t = 24 to 48 hours. Even more significant is the observation that additional PHA was not required after changing the medium, (Chart I, rows 7 and 8) and that the initial stimulus was sufficient to eventuate in mitosis. This interpretation is consistent with the data obtained by Mellman(14), using rabbit anti-PHA serum to inactivate the PHA after initiating mitosis.

No abnormalities of the chromosomes were detected after reversal of mitotic inhibition. Many studies attest to the abnormalities induced in chromosomes by viruses. References to abnormalities induced by mycoplasmas(15) are relatively few despite the fact that accidental contamination of tissue cultures with viable PPLO is commonplace, and its inhibitory effect can often be reversed with antibiotics and by changing the medium(16). Therefore, it should be stressed that the PPLO used in these studies were non-viable in that they could not be recultured either from the inoculum or from the lymphocyte culture at the end of the culture period. Rather than review the evidence for and against viability, allusion will be made to additional work by us which demonstrates inhibitory properties of the PPLO cytoplasm, obtained by osmotic rupture of the organism with distilled water.

Inhibition of mitosis of PPLO suggests many fields of application. *Mycoplasma neurolyticum*(17) produces rolling disease of mice by ischemic necrosis of the cerebellum and cerebral hemispheres. It is also known that some PPLO such as Eaton agent or *Mycoplasma pneumoniae* can cause hemolysis(18). Inhibition of mitosis may also cause pathology and it would be reasonable to hypothesize that *Mycoplasma hominis* type I, whose incidence is increased in chronic cervicitis(19), contributes to the pathogenesis of that disease by inhibiting re-epithelization of the cervix.

At the Second Conference on Biology of the Mycoplasmas under the auspices of the New York Academy of Sciences (May 1966), there were reports on the incidence of PPLO in leukemia, infectious mononucleosis, arthritis, etc. Implicit in all of these reports was the suggestion that the culturing of PPLO had significance regarding the etiology of the disease in some unknown manner. The observation reported here may give impetus to studies elucidating the possible pathogenetic role of PPLO in one or more of these diseases.

Summary. Sufficiently concentrated nonviable *Mycoplasma hominis* type I suspensions will inhibit the mitogenic action of phytohemagglutin in lymphocyte cultures. The inhibition can be reversed by removing the PPLO as late as 48 hours after inoculation. Mitosis can be started with PHA, stopped by PPLO, and restarted without additional PHA by changing the medium.

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Inhibition of Viral Replication by Interferons with Different Molecular Weights.* (31606)

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It has been reported that Newcastle disease virus (NDV), when inoculated into mice or cell cultures, stimulates the appearance of interferon with a molecular weight of about 30,000(1,2). In contrast, in mice injected with *E. coli* endotoxin(2) or statolon(3), an anionic polysaccharide from *Penicillium stoloniferum*, the interferon which appears in the circulation has a molecular weight of about 90,000. Except for their molecular weights, it has been impossible to distinguish the inhibitors produced in mice by nonviral stimuli from the interferon produced by virus (1,2,3). An attempt was made in our laboratory to determine the rate of adsorption of the different molecular weight mouse interferons to homologous cells. In agreement with the results of Buckler *et al*(4), we were unable to show the disappearance of interferon activity from fluid exposed to large numbers of mouse cells. In light of these indications that interferons can render cells resistant to virus in the absence of detectable uptake, it was decided to study the rates at which cells develop resistance to virus challenge after exposure to interferons with markedly different molecular weights.

Materials and methods. Cell cultures and media. L-cells (clone 929) were grown in 32 oz prescription bottles for 7 days with one change of Eagle's medium (MEM) supplemented with 10% calf serum. For interferon assays, all dilutions were made in Eagle's medium supplemented with 4% calf serum.

Interferon was produced in L-cells or in mice as follows. L-cell cultures were inoculated with the Herts strain of Newcastle disease virus (NDV) to give an input multiplicity of 5 to 10 infective particles per cell. After 1 hour of adsorption at 37°C, the inoculum was removed, the cell sheet was washed once, and fresh medium without serum was added to each bottle. After incubation at 37°C for 24 hours, the fluid was removed and clarified by low speed centrifugation. The fluid was brought to pH 2 with 0.1 N HCl, held for 5 days at 5°C to destroy infective

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