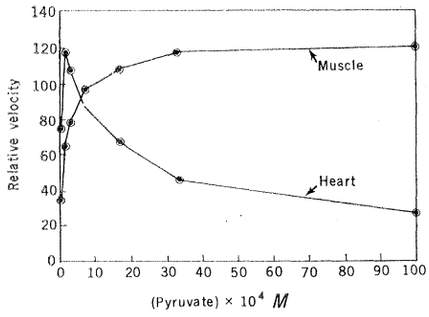

Fig. 2. The effect of pyruvate concentration on *Limulus* D-lactate dehydrogenase catalyses. The initial rate of loss of reduced diphosphopyridine nucleotide (DPNH) absorption at 340 nm was measured as a function of varying pyruvate concentration. Three-milliliter reaction mixtures contained the following concentrations: 0.117 m*M* DPNH (from P-L Biochemicals), and 13.9 m*M* potassium phosphate, *p*H 7.5.

differences were observed between chelicerates and the other arthropods, and between *Nereis* and other annelids.

Analyses of the molecular weights of the various D-lactate enzymes by the Sephadex G-100 method indicate that their weights are close to that of the purified enzyme from *Limulus* muscle, whereas those of the L-lactate enzymes of the invertebrates are roughly the same size as those of the vertebrate lactate dehydrogenases.

In each animal, the lactate dehydrogenases are specific for only one isomer of lactate. This suggests that both the L- and D-lactate enzymes may have arisen from mutation of one gene locus; and we hope to determine this development by further study. The finding of the D-lactate enzyme in invertebrates may add a useful parameter for evaluating phylogenetic and taxonomic schemes for invertebrates.

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Mycobacterium tuberculosis in Macrophages: Effect of Certain Surfactants and Other Membrane-Active Compounds

Abstract. *Some compounds, not directly inhibitory or enhancing, nevertheless influence growth of tubercle bacilli in macrophages in cell culture. They include certain surfactants whose effects can be varied by their structural design. The compounds are probably stored in cell lysosomes. They can interact with various membranes to affect permeability. The anti- and protuberculous surfactants differ in such interaction and also in effect on lysosomal enzyme activity in infected macrophages. A link between the effect on lysosomal membranes and on tuberculous infection is suggested.*

Some compounds suppress or enhance experimental tuberculosis through host-mediation; correlated effects on bacterial multiplication in cell-free medium have not been shown. These compounds include cortisone (*1*), the trypanocidal sulfonated naphthylamine derivative suramin (*2*), and certain nonionic surface-active polyoxyethylene ethers (*3*, *4*) analogous to Triton WR-1339 (*5*). The members of one series of the surfactants [prepared by Cornforth and his colleagues (*3*)] are polyethylene glycol ethers of a *p-tert*-octylphenol-formaldehyde cylic tetramer, and are referred to as HOC-*x* (HOC indicates the constant phenolic nucleus and *x* the average number of ethylene oxide units per phenolic group, which can be varied). The value of *x* and the consequent lipophilic-hydrophilic balance is crucial in determining whether the compound shall suppress (low values) or enhance (high values) murine tuberculosis, or be inactive (intermediate values).

Since this type of surfactant can enter macrophages in the living animal (*6*), and these white cells are a main site of attack in tuberculosis infection, I have now studied whether the contrasting effects could be reproduced when the compounds were applied directly to isolated macrophages infected in tissue culture, or whether participation of the intact host (for example, through the known complex disturbances in lipid metabolism) was essential. Growth of tubercle bacilli in macrophages had been unaffected if WR-1339 was added to the cell culture, although it was inhibited if the cells were from animals that had been treated previously with this compound (*7*); but I suspected that this might be due to the low concentration tolerated in the culture. I used the less toxic HOC-12½ (Macrocyclon) and HOC-60 (mean molecular weights about 3,100 and 11,500), representative respectively of antituberculous and protuberculous (growth-accelerating) activity in the animal (*8*).

Macrophages were obtained from the unstimulated peritoneal cavities of normal mice (P strain) and were maintained as monolayers on glass coverslips in Leighton tubes containing 1 ml of medium suitable for long-term survival (*9*). The tubes were gassed with 5 percent CO_2 in air, closed tightly with silicone rubber bungs, and incubated at 37°C. The medium was changed (with gassing) every 10 to 14 days. Generally, a few days after start of incubation, the external medium was withdrawn and the cell layers were infected by exposure, in the Leighton tubes, to a well-dispersed suspension of bacilli for 2 hours at 37°C. *Mycobacterium tuberculosis,* human strain H37Rv, whose original virulence had become somewhat attenuated, was the strain used mainly, well-grown surface subcultures on Proskauer and Beck liquid medium being homogenized and filtered through paper. The concentration of the suspension was adjusted so as to give a light infection initially, with a mean of 0.5 to 1.0 bacillus per cell. After infection, free bacilli were removed by washing, and the wash fluid was replaced by the usual medium—with the addition of streptomycin (5 unit/ml), to prevent extracellular bacterial multiplication, and of one of the agents (Macrocyclon,

2 m*M*, or HOC-60, 0.5 m*M*, that is, each at 6 mg/ml). Control tubes received 0.9 percent NaCl in a volume equal to that of the surfactant solution. If the experiment lasted long enough to require a further change of medium, the agent was usually again included. Assessment of intracellular bacterial multiplication was made at appropriate intervals from counts obtained by total recovery from the whole culture (*10*); or it was made from the frequency distribution of acid-fast bacilli in the cells on the cover slip, together with the parallel enumeration of heavily infected cells shed into the medium (*11*).

Macrocyclon (6 mg/ml) in the culture medium just after infection markedly inhibited intracellular bacterial multiplication during the next 2 weeks or so, after which there might be an "escape" from this effect; reintroduction of this agent at each change of medium could maintain inhibition for at least 4 weeks of macrophage culture life. On the other hand, HOC-60 (6 mg/ml) caused a striking acceleration of intracellular bacterial growth (see Figs. 1 and 2). Parallel with these differences, the infected cell layers in the Leighton tubes containing HOC-60 showed gross deterioration after 8 to 12 days of incubation, as compared with a period of 2 to 3 weeks required for the infected untreated cultures. In the tubes containing Macrocyclon, the layers were in good condition for at least 4 weeks if this agent was reintroduced at each change of medium. The action of Macrocyclon is bacteriostatic rather than bactericidal (see Fig. 1), which supports the results of bacillary counts in lung homogenates of treated mice (*12*); a first application of Macrocyclon to macrophage cultures 2 weeks after infection failed to affect the course of multiplication. In additional experiments, WR-1339 (1.3 m*M* in the medium, that is, at 6 mg/ml) was toxic to the macrophages; the highest concentration tolerated (0.25 mg/ml) did not inhibit [confirming Mackaness (*7*)].

Thus the protuberculous as well as the antituberculous effects in vivo of the two HOC surfactants are entirely reproducible in tissue culture, and the study system becomes simplified to drug-cell-bacterium. In the medium alone (and without streptomycin) tubercle bacilli grew well and in "corded" manner in the presence both of Macrocyclon and of HOC-60 (6 mg/ml). Even at 60 mg/ml in a cell-free bacteriological medium, the two agents showed no clear difference in their effects on

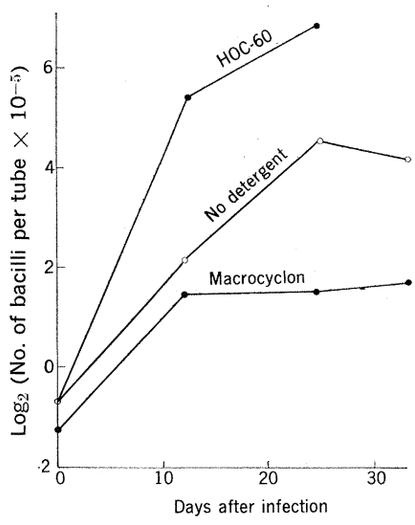

Fig. 1. Growth of *Mycobacterium tuberculosis* in mouse macrophages containing surfactants Macrocyclon or HOC-60, or without agent. Counts of total bacilli recovered from culture tubes (that is, present in cell layers or shed into medium) at different periods after infection.

growth. A cell factor does therefore appear to be implicated in the mechanism.

In uninfected cultures of macrophages with either surfactant added to the medium, the cells swelled progressively over several weeks; the refractive index of the cytoplasm changed and the dry mass (determined by interference refractometry) increased. Changes occurred gradually in the appearance (as seen by phase-contrast microscopy) of cytoplasmic particles of the living macrophages; in the fluorescence pattern when these particles were vitally stained with purified acridine orange (*13*), changing from orange particulate to a diffuse greenish color that suggests diminished entry of the dye into, or a leak from, surfactant-containing lysosomes; and in the pattern of acid phosphatase activity (Gomori method, with prior cell fixation), from the normal heavily stained granules of various sizes to packed, fine, dark granules or a stained "net curtain" appearance. Finally, electron micrographs showed a great increase of large membrane-bound vesicular structures resembling lysosomes, at least some of which contained acid phosphatase (*14*). These structures are similar to those described in the cells from livers of rats injected with WR-1339, which is known to accumulate in (secondary) lysosomes (*15*). By some of these techniques differences between Macrocyclon and HOC-60 were detected, but the main indication is that both are stored in macrophages, probably in association with the lysosomes.

In spite of these marked changes, uninfected macrophage cultures maintained in medium containing either agent at 6 mg/ml remained in good condition for at least a month, judged by appearance, vital staining with neutral red, and ability to phagocytose tubercle bacilli normally with the characteristic results on multiplication. Moreover, most of the changes were slowly reversed in 1 to 2 months after transfer to plain medium.

The probable association of these surfactants with macrophage lysosomes should provide opportunities for membrane effects, such as those noted on the permeability of liver lysosomes when WR-1339 was injected into rats—judged by the rate of release of acid hydrolases from these particles after isolation and exposure to certain stimuli (*15*). Indeed, there is some evidence of differences in membrane activity between our antituberculous and protuberculous agents. Neither Macrocyclon nor HOC-60 is itself hemolytic, even at concentrations as high as 20 mg/ml after 1 hour at 37°C (*4*). After more prolonged incubation however (for example, for 24 to 48 hours at 0.2 to 1.0 mg/ml), I have found slow lysis of human or sheep red cells with Macrocyclon, but less or none with HOC-60; on the other hand, the lysis of human red cells by cetrimide (0.1 mg/ml) was delayed considerably by Macrocyclon but only very little by HOC-60 (0.2 to 1.0 mg/ml). A more striking difference was shown by Lovelock and Rees (*6*) in the lysis of human red cells after sudden cooling, the antituberculous agents decreasing, but the protuberculous increasing, cell sensitivity to this shock. In some recent preliminary experiments Bangham (*16*), using artificial lipid monolayers, found that Macrocyclon interacted strongly with a cholesterol film to form a complex, whereas HOC-60 interacted poorly; and Macrocyclon dissolved the lecithin of a lecithin film, while HOC-60 had a much less solubilizing action.

To observe the effect on lysosomal enzymes of introducing tubercle bacilli into macrophages previously treated (for 10 to 14 days) with one or the other agent, I used the Gomori stain for acid phosphatase activity in unfixed cells on the coverslips (*17*), which was modified to combine with the Ziehl-Neelsen stain for acid-fast bacilli (*18*). If one presumes that the acid phosphatase reaction product indicates activation of lysosomes, this was more—both

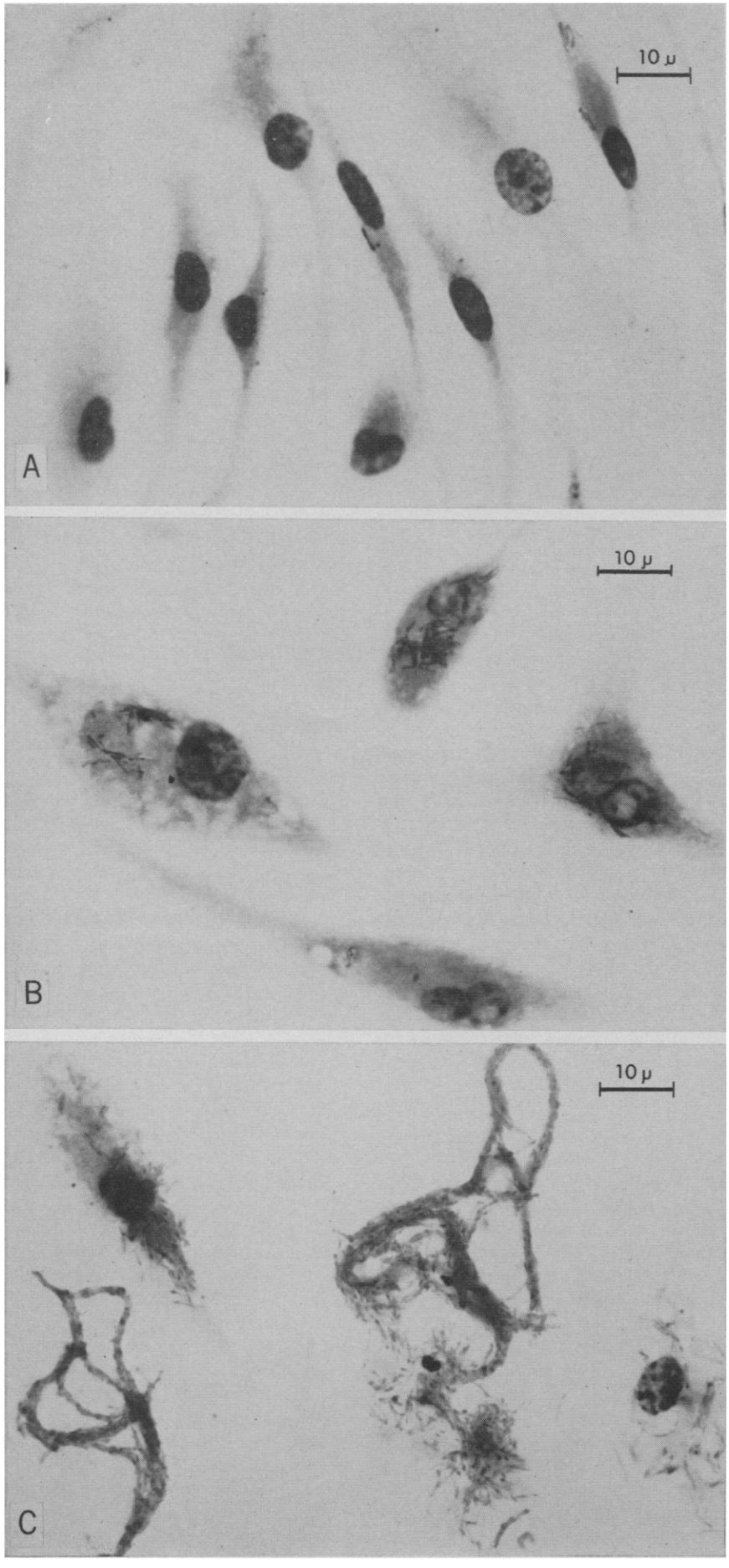

Fig. 2. *Mycobacterium tuberculosis* in mouse macrophages containing Macrocyclon or HOC-60, or without agent. Stained (Ziehl-Neelsen) 9 days after infection (× 1100). (A) Macrocyclon. Few bacilli (no appreciable multiplication compared with day 0). (B) No agent. Moderate multiplication. (C) HOC-60. Marked cord-like growth of bacilli, breaking out of cells.

general and locally associated with the ingested bacilli—after a heavy infection in the macrophages containing HOC-60 than in those containing Macrocyclon; the difference was most evident on the 2nd or 3rd day, that is, before active bacterial multiplication and eventual destruction of cytoplasmic organization occurred in the cells containing HOC-60 (*19*). Lower acid phosphatase activity in association with the antituberculous compound (and it was lower, too, than in infected cells without surfactant) is contrary to what might have been expected if the inhibition of infection were due simply to an excess of digestive or other antibacterial action by lysosomal enzymes discharged into the phagocytic vacuole. In fact, such discharge might promote an environment favorable to growth of the bacilli.

Because of these results with the polyoxyethylene ethers, the effect, on tuberculous infection within macrophages, of some other compounds—selected as also being definitely or probably (i) stored in secondary lysosomes of macrophages or other cells and (ii) membrane-active (*20*)—was similarly examined in tissue culture. Suramin, silica (*11*), dextran (five samples, molecular weights $10^3 \times 2{,}000$, 150, 40, 10, and 5) (*21*), and polyvinylpyrrolidone (molecular weight 12,000), in subtoxic concentrations were markedly protuberculous. With the first two compounds this result corresponds with findings in animals, but with the two polymers it was quite unexpected. The latter had to be in the medium for 2 to 3 weeks before infection, as well as added just afterwards, presumably so as to achieve adequate concentrations. The enhancement of bacterial multiplication by dextran increased with its molecular weight. Since none of these protuberculous compounds had demonstrable growth-promoting action on tubercle bacilli in cell-free medium, their effect within macrophages would seem, like that of the surface-active ethers, to be cell-mediated.

The compounds investigated here have various biological properties. However, it would seem a reasonable hypothesis, albeit from correlative evi-

dence, that association with cell lysosomes, followed by interaction with lysosomal membranes to produce permeability changes, are factors involved in the initial steps of the effects of these compounds on growth of intracellular tubercle bacilli, even though the nature of the interaction may well differ as between the surface-active ethers and the more inert polyvinylpyrrolidone, the charged suramin and uncharged dextran, and so forth. In this connection we have the difference in action on membranes by the long- and short-chained ethers, although so far shown directly only in nonlysosomal systems and after contact in vitro. A possible clue to the mechanism of their contrasting effects on tuberculous infection is offered by the histochemical evidence of differing lysosomal enzyme activity in infected surfactant-treated macrophages. The interpretation of this difference must be speculative at this stage, the possibilities being as diverse as (i) that reduced release of enzyme from lysosomes containing Macrocyclon leaves the acidic environment in the phagocytic vacuoles free to inhibit bacillary growth nonspecifically (*22*), whereas increased release from lysosomes containing HOC-60 produces a situation protective to the bacilli; or (ii) that surfactant-containing lysosomes selectively release into the cytoplasm inhibitory or growth-promoting substances affecting tubercle bacilli elsewhere in the cell (*11*); but a simpler possibility is not excluded, namely (iii) that the agents alter the surface of the bacterium, which is thus made less or more susceptible to attack by cell enzymes (*23*).

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23 August 1968

Red-Cell Transport Defect in Patients with Cystic Fibrosis and in Their Parents

Abstract. *The ouabain-sensitive and the ethacrynic acid–sensitive sodium efflux from erythrocytes of patients with cystic fibrosis are both decreased. Furthermore, the ouabain-sensitive adenosine triphosphatase activity is diminished in the red blood cell ghosts of these patients. Perhaps of greater significance is the fact that ethacrynic acid–sensitive sodium efflux is clearly diminished in the erythrocytes of the asymptomatic parents of these sick children. This defect in sodium transport may be valuable for detecting the heterozygous carrier state.*

There is an elevated concentration of sodium and chloride in the sweat of patients with cystic fibrosis (*1*). Mangos and McSherry (*2*) demonstrated that the sweat from patients with this disease inhibits sodium transport in the duct system of the rat parotid gland. The measurement of ion concentrations in sweat as a marker for the heterozygote state has been reviewed (*3*). Spock *et al.* (*4*) demonstrated that serum from patients with cystic fibrosis and their parents promoted a disorganization of the ciliary rhythm in explants of respiratory epithelium. Danes and Bearn (*5*) reported cytoplasmic intravesicular metachromasia with specific characteristics in skin fibroblast cultures from seven children with the disease and in 13 of the 14 parents. The study of Mangos and McSherry (*2*) motivated us to examine the components of active transport in another cell membrane system, the erythrocytes of patients with cystic fibrosis and their parents.

The mean concentration of sodium per liter of red blood cells in 18 children with the disease was 7.3 ± 0.39 mmole/liter (*6*). This compares well with the mean value in a group of 108 Caucasian controls in our laboratory, with a value of 7.2 ± 0.12 mmole/liter.

Sodium efflux studies were done with erythrocytes from patients and parents of patients, and each study was coupled with a control. In some instances the

Table 1. Active sodium transport and adenosine triphosphatase (ATPase) activity in erythrocytes of patients with cystic fibrosis. The ratio of activities of patients to controls of pumps I and II were significantly different at a $P < .005$; the adenosine triphosphatase activity at a $P < .023$, and the "leak" at a $P < .01$ as calculated by the rank-sum test of Wilcoxon. The definitions of pump I and pump II are those of Hoffman (*8*). $\bar{x}$, mean ± S.E.M.

Pump I	Pump II	"Leak"	Ouabain-sensitive-ATPase
.89	.15	.54	.59
.87	.66	.87	.45
1.02	.63	.83	.34
.66	.31	.71	.92
.92	.64	.59	.64
.88	.41	1.35	1.11
.88	.56	.40	.73
.69	.60	.91	
.89	.50	.52	
.77	.28	.67	
$\bar{x}$ =	$\bar{x}$ =	$\bar{x}$ =	$\bar{x}$ =
.85 ± .03	.47 ± .06	.74 ± .09	.68 ± .10