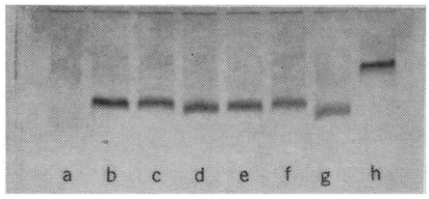

Fig. 1. (*a* to *g*) Serum specimens from subject B.S. taken at different times: (*a* and *b*) specimens 3 weeks apart; (*c* to *g*) samples taken during a glucose tolerance test, at half-hour intervals (*g* is the fasting specimen); (*h*) serum specimen from subject T.C. Electrophoresis in tris-EDTA buffer *p*H 9.0, 3-percent polyacrylamide gel, at 150 volts for 2.5 hours at 26°C; preliminary run for 15 minutes.

in the refrigerator or for weeks in the freezer gave qualitatively identical results. Hyperlipemic serums produced streaked patterns unless subjected to high-speed centrifugation before electrophoresis.

The serum samples were stained, prior to electrophoresis (*2*), with a dye solution consisting of 1-percent lipid crimson (Gurr, London) in diethylene glycol; the dye was filtered before use. The combined serum (200 μl) and dye solution (100 μl) were agitated for 5 minutes and centrifuged (Spinco 152 Microfuge) for 20 minutes. A small amount of precipitated dye was usually visible at the bottom of the tube. The clear supernatant (20 μl) was placed for electrophoresis in a slot 1 cm wide. The supernatant was reddish-purple; if lipoprotein was absent from the sample the supernatant was similarly colored, although no stained band appeared in the pattern.

The vertical cell described by Raymond (*3*) was used. The electrophoresis buffer at *p*H 9.0 contained 50 g of ethylenediaminetetraacetate (EDTA) and 460 g of tris in 45 liters of water. All gel solutions were made up in this buffer. The cell was supported at an angle of 30°, and 50 ml of an 8-percent gel solution [including 0.1 percent each *N,N,N′,N′*-tetramethylethylenediamine (TMED) and ammonium persulfate] was poured into the cell, forming a layer 3 cm deep at the bottom of the gel space. The 8-percent gel supported the 3-percent gel subsequently poured, but did not form part of the electrophoresis pattern. After the supporting gel was fully polymerized, the cell was returned to the horizontal position and 180 ml of 3-percent gel solution was poured into it. The 3-percent gel was allowed to polymerize for 2 hours, the excess gel above the slot form was removed, and the cell was then placed in the upright position. The buffer compartments were filled with buffer, and the slot form was removed. Circulation of the buffer facilitated removal of the slot form.

After a 15-minute preliminary run at 150 volts, 20 μl of the stained serum was placed in each 1-cm slot. Current was then applied at 150 volts with buffer recirculation. The temperature of the cooling plates was controlled by circulating tap water (26° to 30°C). After 2.5 hours, the visible pattern included one or more red bands in the β-lipoprotein region and two diffuse bands in the region of α-lipoprotein and albumin.

For subsequent operations a 3-percent gel was difficult to handle. Therefore a technique was developed to stiffen the gel before removing it from the electrophoresis cell. After completion of the electrophoresis, the buffer was drained, the cell was placed in the horizontal position, the upper cooling assembly was removed, and the excess gel was trimmed from the pattern. The remaining gel was covered with 120 ml of buffer containing 10 percent of cyanogum and 0.1 percent of TMED, the persulfate being omitted. After 45 minutes, 30 ml of 0.5-percent ammonium persulfate was mixed with the gel solution. A cover was placed over the pattern to exclude air and permit the stiffening gel to polymerize uniformly. By this procedure, a gel with strength close to that of an 8-percent gel was produced.

The stained lipoproteins did not migrate satisfactorily in gels of greater than 3.75-percent concentration. In 3-percent gel the pattern exhibited a zone visible about 1 to 2 cm from the origin which reacted with antiserum to β-lipoprotein. In addition there were two diffuse stained zones about 7.5 cm from the origin, one of which was identified as albumin by staining with amido black; the other, slightly slower, was presumed to be α-lipoprotein, which occurs in this position in other electrophoresis media.

In this procedure the β-lipoprotein bands exhibited variations in mobility not heretofore reported. Figures 1 and 2 show these variations in serum specimens from a single individual, and between specimens from different individuals.

In separate experiments with 5- and 8-percent gels in the techniques of Peacock (*4*) diethylene glycol either alone or with lipid crimson did not visibly alter the serum protein pattern in tris-EDTA buffer. However, in buffers containing borate, diethylene glycol caused the appearance of an extra amido-black-staining band near that of transferrin.

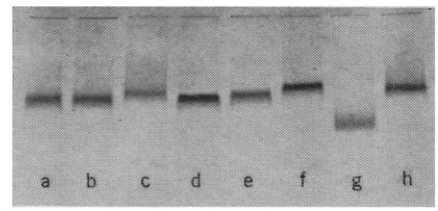

Fig. 2. (*a* to *e*) Serum specimens taken at 2-day intervals from subject N.S.; (*f* to *h*) serum specimens taken at 3-day intervals from subject M.A. Electrophoresis as described for Fig. 1.

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13 October 1965

Chloroquine: Mode of Action

Abstract. *The drug chloroquine is bactericidal for* Bacillus megaterium; *it inhibits DNA and RNA biosynthesis and produces rapid degradation of ribosomes and dissimilation of ribosomal RNA. Inhibition of protein synthesis is also observed, evidently as a secondary effect. Inhibition of DNA replication is proposed as a general mechanism of the antimicrobial action of chloroquine.*

The antimalarial drug, chloroquine (Resochin, *1*), forms a complex with DNA (*2*, *3*) and inhibits in vitro reactions catalyzed by DNA polymerase (*4*), RNA polymerase (*4*), and deoxyribonuclease (*3*). It also inhibits incorporation of P^{32}-orthophosphate into the nucleic acids of two plasmodia (*5*). Although bacteria are relatively insensitive to the drug (*6*) we have found a strain of *Bacillus megaterium* to be susceptible to chloroquine (*7*) and

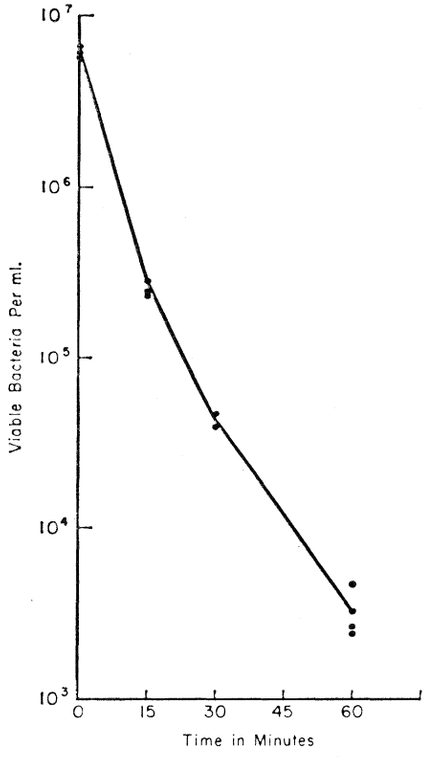

Fig. 1. Decrease in viability of chloroquine-treated *B. megaterium*. The test strain 46-U-1 (furnished by the Department of Bacteriology of our Institute) was grown in Sauton's medium (*16*) in which DL-glutamic acid was replaced by the L-isomer, and 0.2 percent glucose was substituted for glycerol. Chloroquine was added as the hydrochloride (a commercial product) to exponentially growing cultures to a concentration of $10^{-3}M$; samples were removed at intervals for serial dilution plate counting.

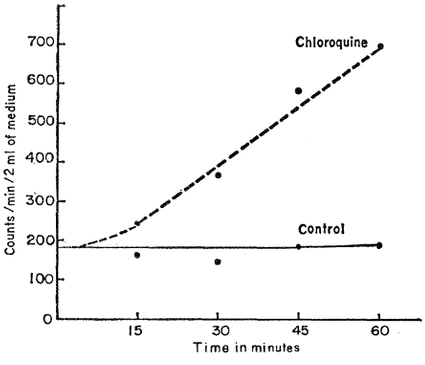

Fig. 2. Release of radioactivity from *B. megaterium* labeled with uracil-C^{14} upon incubation with chloroquine ($1.6 \times 10^{-3}M$). The bacteria were grown for 2 hours in modified Sauton's medium (Fig. 1) containing 332 μg/liter of uracil-C^{14} of specific activity 0.7 μc/μmole; they were then collected and resuspended in fresh uracil-free medium with (top line) or without (bottom line) added chloroquine. Radioactivities were measured in membrane filtrates of samples taken at intervals; a Nuclear-Chicago liquid scintillation counter and a dioxane-based scintillation fluid were used.

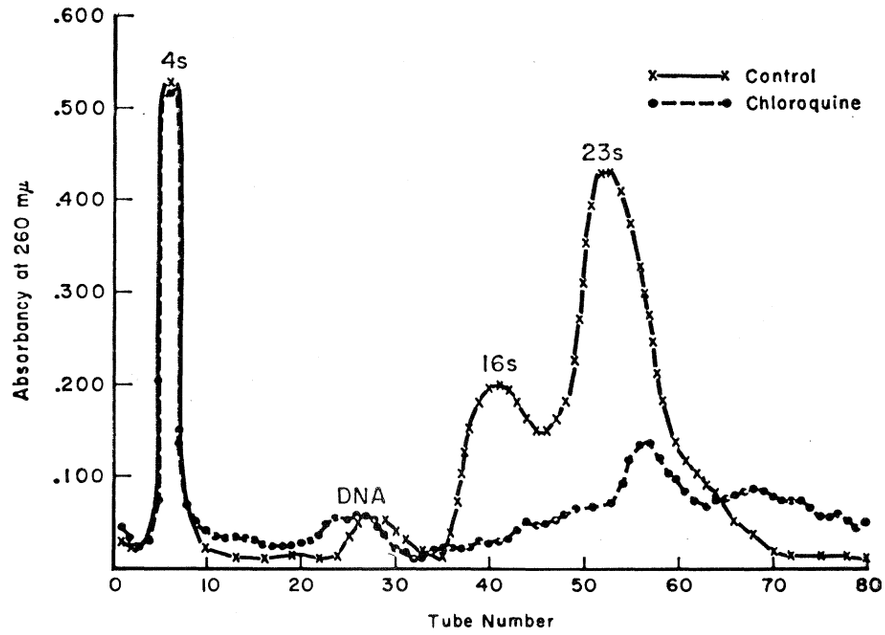

Fig. 3. Chromatographic analysis of nucleic acids of *B. megaterium* incubated for 1 hour with $1.6 \times 10^{-3}M$ chloroquine (solid circles) or without the drug (crosses). Bacteria were collected and disrupted by sudden release from hydrostatic pressure in a French pressure cell (Aminco). Nucleic acids were obtained by phenol extraction (*17*) and chromatographed on a methylated albumin-kieselguhr column (*18*) by elution with a linear gradient of NaCl from 0.5 to 1.0*M*, buffered with phosphate at *p*H 6.7.

have studied the mode of action of the drug with this organism; these studies are the subject of this report.

Addition of chloroquine to cultures of *B. megaterium* in exponential growth resulted in rapid decline in bacterial viability (Fig. 1). During the first 20 minutes after addition of the drug, bacterial density, measured turbidimetrically, increased approximately 10 percent and then remained constant. Chemical analyses for DNA (*8*) and RNA (*9*) of bacteria in samples of culture taken at intervals showed not only that nucleic acid biosynthesis was blocked by chloroquine but also that the bacteria lost approximately 10 percent of their total RNA during 1 hour of exposure. When global RNA was labeled with uracil-C^{14}, radioactive label was lost progressively from chloroquine-exposed organisms but not from drug-free control bacteria (Fig. 2). During 1 hour of incubation, this loss amounted to 10 percent of the total radioactivity present; the result is in agreement with the results of chemical analyses for RNA.

Chromatographic analysis of the phenol-extracted global nucleic acids revealed that *B. megaterium*, upon exposure to chloroquine for 1 hour, dissimilated all 16*S* and most of the 23*S* ribosomal RNA's; transfer-RNA and DNA were not degraded (Fig. 3). Evidently only a small fraction of the ribosomal RNA was degraded to products sufficiently small to be excreted by the test bacteria; most of the ribosomal RNA remained inside the bacterial cells in the form of products of no preferred molecular size. This dissimilation of ribosomal RNA suggested that the ribosome particles themselves were breaking up. Figure 4 depicts the time course of ribosome degradation in chloroquine-exposed *B. megaterium* and indicates that the organisms degraded 70 percent of their ribosomes during the first 10 minutes of drug action.

When phenylalanine-C^{14} was supplied to test cultures immediately before addition of chloroquine and the radioactivities of the bacterial proteins were determined at intervals by a membrane filter technique (*10*), no significant quantities of the amino acid were incorporated. The conclusion that protein synthesis was inhibited is in accord with the observation that bacterial density failed to increase significantly after addition of chloroquine.

Oxidation of glucose by washed suspensions of *B. megaterium* as measured manometrically (*11*) was inhibited by only 10 percent at a bactericidal concentration of chloroquine of 7.8×10^{-4} mole/liter. A graphic probit transformation (*12*) of inhibition of oxygen

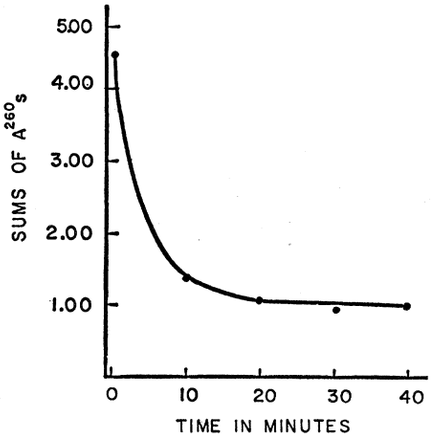

Fig. 4. Decrease in ribosome content of *B. megaterium* during exposure to chloroquine ($1.6 \times 10^{-3}M$). Samples (1000 ml) of experimental mass cultures were taken at intervals, the bacteria were collected and disrupted as described in the legend to Fig. 3, and the samples were clarified by low-speed centrifugation and dialyzed for 24 hours at 4°C against a buffer mixture introduced by Nirenberg and Matthaei into experimentation with ribosomes (*19*). The samples were then subjected to molecular sieve analysis (*20*) by being placed on columns of Sephadex G-100 and eluted with fresh buffer. Relative quantities of ribosomes in eluted fractions were estimated spectrophotometrically (wavelength 260 mμ), and the sums of the absorbancies (A^{260}s) in fractions comprising entire ribosomal peaks were plotted as a function of the time of bacterial exposure to chloroquine.

consumption as a function of chloroquine concentration yielded a straight line with a value of ED_{50} (the 50-percent effective dose) of 760 μg/ml, that is, 2.4×10^{-3} mole/liter. Evidently the inhibitions of macromolecular biosyntheses we report are not results of a general anabolic failure owing to blocking of electron-transfer reactions; oxidative phosphorylation is insensitive to chloroquine in cells whose growth is inhibited by the drug (*13*).

Our finding that DNA synthesis in bacteria is inhibited in vivo by chloroquine is in essential agreement with observations that incorporation of radiophosphate into nucleic acids of plasmodia is inhibited by this drug (*5*); blockage of DNA replication per se explains, in our opinion, the bactericidal effect of chloroquine.

The breakup of ribosomes and the dissimilation of ribosomal RNA were unexpected findings, although another instance is known (*14*) in which blocking of DNA replication was accompanied by similar phenomena. Degradation of ribosomes and their RNA in our experiments with chloroquine explains the observed net loss of RNA from *B. megaterium* as well as the failure of protein synthesis and may have contributed to the bactericidal effect of the drug upon this organism.

We propose that inhibition of DNA replication, based upon a direct action of the drug on DNA, is the general mode of antimicrobial action of chloroquine. This idea has certain implications for observations of natural or acquired resistance to the drug. The molecular architecture of double-stranded DNA is evidently universal, and susceptibility or resistance to chloroquine cannot be explained on the basis of structural or compositional differences between the DNA's of susceptible or resistant cells. It is more likely that susceptibility to chloroquine, like that to actinomycin D (*15*), is based upon the capacity of susceptible cells to permit passage and accumulation of critical concentrations of the drug while natural or acquired resistance may be results of impermeability or of an impaired concentration mechanism. This is borne out by two observations. (*i*) Chloroquine-exposed and packed cells of susceptible *B. megaterium* contained ten times as much of the drug as did identical volumes of packed, chloroquine-resistant *B. cereus* (*11*). (ii) The DNA-polymerase reaction in cell-free experiments in vitro is highly susceptible to chloroquine (*4*) in spite of the fact that the priming DNA as well as the enzyme has been prepared from *Escherichia coli* which is resistant to chloroquine (*6*).

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Subcellular Sources of Luminescence in Noctiluca

Abstract. *The perivacuolar cytoplasm of* Noctiluca miliaris *contains approximately 10^4 microsources of luminescence, with dimensions of 0.5 to 1.5 microns, which exhibit marked fluorescence with ultraviolet excitation. Local invasion by an action potential elicits light emission (microflashes) from these sources with a coupling latency of about 2 milliseconds. Magnitudes of the microflash vary directly with the dimensions of the source. Time courses of the microflash resemble that of the macroflash emitted by the whole cell but have somewhat shorter time constants. The small discrepancy in duration between micro- and macroflash can be explained by the 5- to 10-millisecond asynchrony of microsource triggering that results from the conduction time of the action potential. Reversible gradations in amplitude of the macroflash, as from potentiation or fatigue, result from parallel summation of graded changes in microflash intensity. Thus the macroflash gives a reasonably true picture of the subcellular kinetics of luminescence.*

The luminescent flash of the dinoflagellate *Noctiluca miliaris* is triggered by an all-or-none action potential that propagates nondecrementally in the complex peripheral layer of cytoplasm at a rate of about 60 μ/msec (*1*, *2*).