of distilled water. The bridge of 0.03*M* aluminum lactate buffer was adjusted to *p*H 2.4 with lactic acid. Two sheets of filter-paper strips were inserted 4 cm from the anodal end of the gel (12 by 16 by 0.6 cm), and 30-μl samples were applied. Horizontal electrophoresis was performed at a constant voltage of 150 volts for 12 to 13 hours. The gel surface was covered with Saran wrap to prevent evaporation, and a box containing ice was placed on the gel. During electrophoresis the gel current intensity was approximately 45 ma. Prior to being stained, the 6-mm thick gel was cut into 2-mm slices; the upper slice was discarded and the lower two slices were stained by the method developed by Sung and Smithies (*4*) for arginine-rich proteins.

Figure 1 shows the Pm band located between bands Pa and Pb. The Pm phenotypes are determined by the presence (+) or absence (−) of this middle-band protein. Figure 2 shows that the densitometric tracing pattern of the Pm(−) sample lacks the peak typical of Pm(+) samples.

In Fig. 3, which shows a gel used for the electrophoresis of parotid saliva samples from five individuals, the Pb proteins have migrated out from the gel frame. By this method, the migration distance of the Pm band was increased, and the separation of the Pm zone was improved. The band that migrates after the Pm band can be recognized in both Pm(+) and Pm(−) samples and simplifies Pm typing. Saliva collected from Pm(−) individuals at different times of the day and on different days never revealed the Pm protein. The Pm protein is also observed in whole saliva and seems to be fairly stable compared with other salivary proteins.

The Pm(+) phenotype has been observed in both of the two racial populations studied, Japanese and Chinese. Among 195 samples from the Japanese population, 120 (61.5 percent) were of the Pm(+) type and 75 (38.5 percent) were of the Pm(−) type. Among 20 samples from the Chinese population, ten were Pm(+) and the other ten were Pm(−).

Family studies including 28 families and 59 offspring were done to test the genetic hypothesis. Of ten matings where both parents had the Pm(+) phenotype, there were 24 Pm(+) and five Pm(−) offspring. Of eight matings where both parents were Pm(−), all 11 offspring had the expected Pm(−) phenotype. Of ten matings where one parent was Pm(+) and the other Pm(−), there were 14 Pm(+) and five Pm(−) offspring. These data are consistent with the hypothesis that the Pm(+) phenotype is an autosomal dominant trait. From this hypothesis, estimates of the frequencies of the genes that determine these phenotypes in Japanese were, for Pm^+, .38 ± .03, and for Pm^-, .62 ± .03.

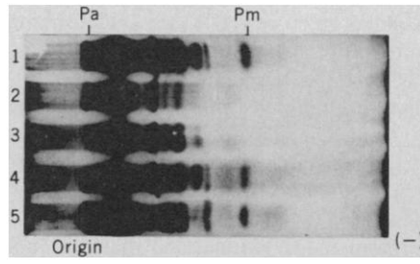

Fig. 3. Electrophoresis of parotid saliva from five individuals by our modified method. For the accurate typing of Pm phenotypes, saliva samples were concentrated approximately ten times and horizontal electrophoresis was performed at a constant voltage of 150 volts (45 ma) for 12 to 13 hours. The Pb proteins have migrated out from the gel. The broad band indicated by a black spot migrates after the Pm band and is recognized in both Pm(+) and Pm(−) samples. Each channel contains a sample from a different individual, and the Pm and Pa phenotypes of each sample are, respectively, channel 1, Pm(+) and Pa(+); channel 2, Pm(−) and Pa(+); channel 3, Pm(−) and Pa(−); channel 4, Pm(+) and Pa(−); and channel 5, Pm(+) and Pa(+).

Table 1 shows the distribution of Pm and Pa phenotypes in 145 unrelated Japanese typed for both marker systems. Estimates of the frequencies of the genes determining Pa^+ and Pa^- individuals in this sample were .23 ± .03 and .77 ± .03, respectively. The observed distribution of 58 Pa(+) (59.0 expected) and 87 Pa(−) (86.0 expected) is consistent with Hardy-Weinberg equilibrium (χ^2 = 0.029; $.80 < P < .90$). When this sample was tested for equilibrium with respect to the two loci, the observed distribution of Pm and Pa phenotypes is compatible with expected equilibrium frequencies (χ^2 = 1.006; $.30 < P < .50$). In addition, all 145 samples were of the Pb (1-1) type (*1*). From these results we concluded that Pm is not related to Pa or Pb.

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13 October 1976

In vitro Growth of *Mycobacterium lepraemurium*, an Obligate Intracellular Microbe

Abstract. *By using an ultrasensitive technique to measure adenosine triphosphate in terms of functional biomass, we have confirmed that* Mycobacterium lepraemurium *(the agent of rat leprosy and a classical obligate intracellular microbe) grows in vitro in the Nakamura system. By using a sulfhydryl-containing medium that occupies 65 to 75 percent of the culture tube volume, together with the five supplements recommended by Nakamura, we have obtained growth rates some eight times above the original. The new physicochemical environment and the use of adenosine triphosphate as an index of energy status in the presence and absence of growth provide a basis for investigating the physiology and growth of other noncultivated microbes.*

For 103 years (1873 to 1976) the inability to cultivate *Mycobacterium leprae* in vitro has been a major bottleneck in leprosy research. For 73 years (1903 to 1976) a second "obligate intracellular" mycobacterium, *M. lepraemurium* (the agent of rat leprosy), has been used by many investigators as an interim model because (i) its growth potential is higher than that of *M. leprae* and (ii) it is transmissible in rats and mice. The decades of attempts to cultivate these bacteria in vitro suggest that success will require more than the testing of different combinations of nutrients and will depend in part upon the application of new principles or conditions.

Using firefly bioluminescence, we have developed (*1*) an ultrasensitive method for measuring adenosine triphosphate (ATP) in terms of functional biomass (the percentage of ATP in the suspension or culture) in unwashed host-grown bacteria and in bacteria grown in

vitro. This technique has enabled us to confirm Rightsel and Wiygul's (*2*) discovery that the Hawaiian strain of *M. lepraemurium* is capable of extracellular growth in diffusion chambers implanted in the peritoneal cavities of mice. It has also enabled us to demonstrate that increases in functional biomass parallel increases in total biomass, and that this "obligate intracellular" parasite is able to produce growth-competent cells under conditions that are reproducible in vitro. Meanwhile, Nakamura (*3*) has reported more rapid growth of *M. lepraemurium* in a system he developed to promote the Hart-Valentine (*4*) phenomenon of elongation (unbalanced growth) in vitro. The most important feature of the Nakamura system is that much of the air in the culture tubes (13 by 105 mm) is displaced by filling the tubes to 67 percent of capacity with NC-5 medium. This medium consists of an autoclaved, Kirchner-type, semisynthetic base [EK; see (*3*)] and five aseptically added supplements: α-ketoglutarate, hemin, cysteine, cytochrome c, and goat serum.

In this report we describe data that confirm those of Nakamura. Our experiments demonstrate the almost disastrous decline in the energy production of intracellular microbes that are transforming into more competent cells of the type that grow in vitro, and enable us to outline preliminary improvements in the system. Gains in total biomass were determined microscopically from counts (*5*) of cell units multiplied by the average length of these units.

Both the functional and the total biomass of *M. lepraemurium* showed approximately sevenfold increases during 8 weeks in the original Nakamura system. In the EK base the functional biomass decreased precipitously while the total biomass remained constant. The lowest point on the curves for NC-5 medium at 0.4 week (3 days) depicts the period required for the transition of host-grown (type 1) cells into (type 2) cells adapted to growth in vitro. The special merits of the supplements in the NC-5 medium were apparent within 3 days. They reduced the early physiologic lag by 50 percent, that is, to 75 percent of the original functional biomass, and activated the growth of cells of type 2. By day 14 the cells had regained the original energy (ATP) levels. Within 2 weeks, fetal calf serum was unmistakenly superior to the goat serum recommended by Nakamura. Figure 1B shows the approximate 2-week lag in the expansion of total biomass and the parallelisms between increases in functional and total biomass. The final cells were 1.4 times longer than the original cells.

Figure 2 illustrates the 17-fold increases in cell populations attained after 4 weeks in the optimized Nakamura system. The gains were achieved by means of 25 percent air space, freshly dissolved pyruvate, inactivated fetal calf serum, and 0.7 m*M* cysteine or thioglycolate. These improvements did not prevent the failure of growth after 6 weeks. Optimal oxidation-reducation potentials produced by agents such as ascorbic acid showed that the growth of *M. lepraemurium* depended upon the presence of the sulfhydryl group. The presence of cystine was slightly more inhibitory than the absence of sulfhydryl groups.

This confirmation of Nakamura's work by means of a technique that measures losses of functional biomass as readily as gains opens a path toward the cultivation in vitro of medically important "obligate intracellular" organisms. In particular, Nakamura's definition of a

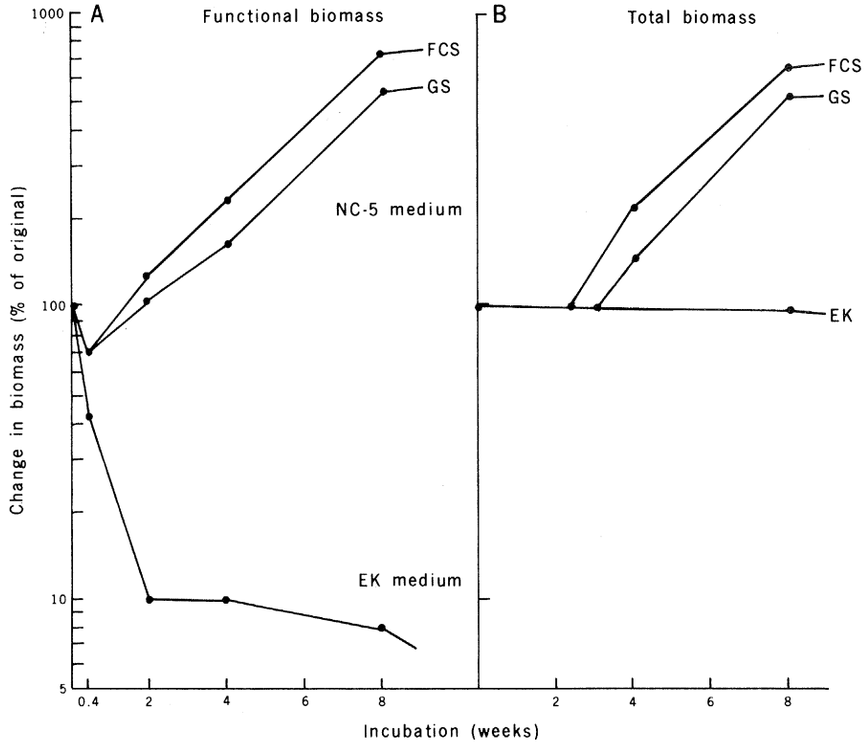

Fig. 1. Growth of *M. lepraemurium* in Nakamura's system. (A) Functional biomass is described by the percentage of ATP in the culture. (B) Total biomass is determined from cell numbers multiplied by average cell length (*FCS*, fetal calf serum; *GS*, goat serum). The culture vessels were screw cap tubes (13 by 100 mm) with a total volume of 9.2 ml. Each tube contained 6 ml of medium, and 35 percent of the volume was filled with air. Cultures were maintained at 30°C. The average number of bacteria per milliliter was 1×10^6; the number per culture was 6×10^6. Four of the conditions described by Nakamura are less than optimal: 35 percent air space; refrigerated pyruvate; goat serum; and 0.4 m*M* cysteine pipetted into the medium, this medium then being pipetted into culture tubes.

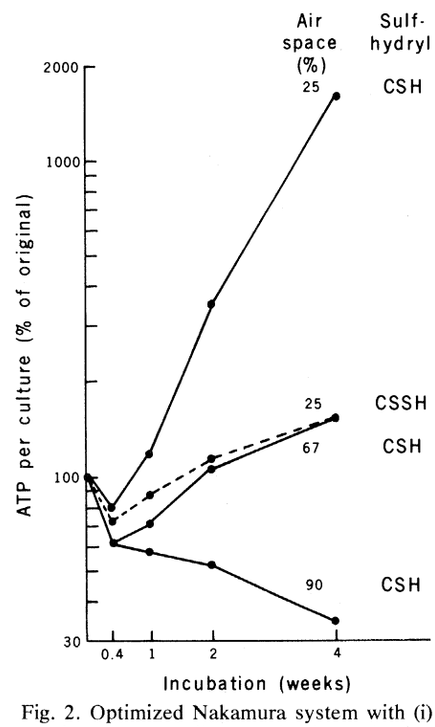

Fig. 2. Optimized Nakamura system with (i) restricted oxygen, (ii) low oxidation-reduction potential, and (iii) the presence of sulfhydryl groups. The average number of bacteria per culture was 7×10^6. The air space is expressed as a percentage of volume. Innovations: optimum air space 25 percent; freshly prepared pyruvate; inactivated fetal calf serum; and inoculated medium pipetted into tubes containing sterile 0.7 m*M* cysteine (*CSH*) or cystine (*CSSH*).

physicochemical environment that can substitute for the intraphagosomal milieu seems fundamental to further successes. The Nakamura system is an example of the serendipity that arises in basic research. It seems unlikely that seven key factors—oxygen restriction, the presence of sulfhydryl groups that meet the specific sulfur requirements of *M. lepraemurium*, and five supplements—could have been chosen and aligned without the guidelines provided by the phenomenon of elongation. Nakamura's choice of organism was fortunate, because *M. leprae* and other noncultivated organisms do not afford morphologic evidence of active systems in vitro. For such organisms, biochemical indicators of physiologic status will be needed to measure the effects of favorable factors in the absence of growth. Metabolic methods such as respiration and hydrogen transfer capacity (*6*) proved of limited use because of the leakiness of intracellular microorganisms. By the time such organisms have been washed free of host components, they retain only vestiges of their normal metabolic activity (*7*). A merit of measuring ATP is that data can be obtained immediately from unwashed host-grown organisms. Such data have here been shown to indicate (i) the lowest point of energy production by type 1 (host-adapted cells); (ii) the point at which ATP production again increases, which shows that type 2 cells have become functional; (iii) the time required for the organism to produce energy in vitro in the same quantities as in vivo; and (iv) the expansion of cell populations thereafter.

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Stimulation of in vitro Translation of Messenger RNA by Actinomycin D and Cordycepin

Abstract. *Actinomycin D and cordycepin were tested for their effect on translation in the wheat germ embryo extract and reticulocyte lysate assays for in vitro protein synthesis. Both drugs were found to stimulate the incorporation of ^{35}S-labeled methionine into protein up to threefold as compared to control assays. This was true for synthesis directed by murine myeloma polyadenylate–containing RNA, tobacco mosaic virus RNA, and endogenous reticulocyte messenger RNA.*

Actinomycin D and cordycepin (3′-deoxyadenosine) are inhibitors of transcription that have been widely used in studies of gene regulation. In order to eliminate ambiguity in interpreting the results of several kinds of experiments—for example, determinations of the half-life of messenger RNA (mRNA) (*1*) and superinduction studies (*2*)—it is necessary to assume that these compounds do not significantly affect translation. However, this assumption has been tested for actinomycin D with conflicting results. Revel and Hiatt (*3*) observed as much capacity for protein synthesis in rat livers exposed to actinomycin D for 40 hours as in control livers. Palmiter and Schimke (*4*) analyzed the effect of actinomycin D on ovalbumin mRNA translation and found an increase in the rate of in vivo elongation with a maintenance of polysome size. These results imply an increase in the rate of polypeptide initiation and thus in translation in the presence of actinomycin D. Another study (*5*) showed a contrary result, an actinomycin D–induced decay in the rate of protein synthesis in HeLa cells which was attributed to an effect on the association of mRNA with ribosomes. Sargent and Raff (*6*) investigated the effect of actinomycin D on protein synthesis and polyadenylation capacities of mRNA in activated enucleate sea urchin eggs and found no effect of the drug on either process; findings in the sea urchin system may not be applicable to other systems because of the conditions of the mRNA stability and the permeability of sea urchin eggs. A problem common to all but the last study listed above is that it is nearly impossible to uncouple transcription and translation in intact cells.

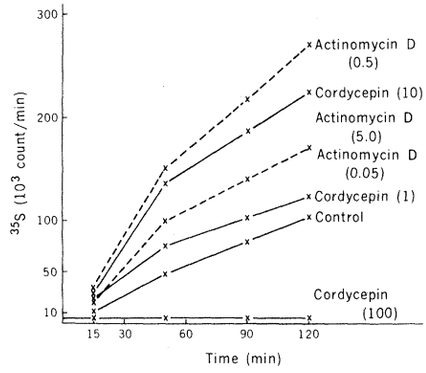

Fig. 1. Incorporation of ^{35}S-labeled methionine into protein by myeloma mRNA in wheat germ embryo extracts as described below. Actinomycin D and cordycepin were added in 1-μl volumes (at concentrations indicated) to 25-μl assays to which was added 1 μg of myeloma poly(A)–containing RNA. Portions (5 μl) were removed at indicated times, and incorporation of isotope into material precipitable by trichloroacetic acid was determined. Myeloma mRNA was isolated from RPC5.4 cells by proteinase K (EM Laboratories) digestion of supernatants from which the mitochondria had been removed (*9*); the supernatants were then chromatographed on oligo(dT) cellulose (*10*). The RNA was translated in a wheat germ extract system as described (*11*), except for the following modifications: 28 m*M* Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), *p*H 7.1, in place of tris; 40 μ*M* spermine was included in the assay mixture; 80 m*M* K^+ in place of 120 m*M* K^+. Incubations were at room temperature.

Our studies were designed to answer two questions: (i) Do transcriptional inhibitors such as actinomycin D and cordycepin affect in vitro translation? And, if so, (ii) are the protein products affected differentially?

We have studied directly the effect of actinomycin D and cordycepin on protein synthesis by using in vitro translation systems. Two types of extracts have been used to investigate the effect of the drugs on protein synthesis: the wheat germ embryo extract system and the reticulocyte lysate system. The wheat germ system was chosen because it has very low endogenous incorporation and is very highly stimulated by many prokaryotic and eukaryotic mRNA's. We have used mouse myeloma RNA that contains polyadenylate [poly(A)] and RNA from tobacco mosaic virus (TMV) to direct synthesis in wheat germ assays and used translation of endogenous mRNA's in reticulocyte lysates. Our experiments demonstrate that actinomycin D and cordycepin stimulate in vitro incorporation of ^{35}S-labeled methionine into polypeptides directed by TMV, myeloma, and endogenous reticulocyte mRNA up to threefold as compared to the controls.

Each drug increased both the rate and extent of incorporation of [^{35}S]methi-