Reports and Letters

Submerged Culture of Micrococcus Lysodeikticus for Large-Scale Producton of Cells

Published methods for the production of large amounts of cells of *Micrococcus lysodeikticus* (*1*) for the isolation of bacterial catalase have consisted of surface culture techniques. For obvious reasons these methods are not very satisfactory or convenient. We have, therefore, explored the possibility of growing this organism as a submerged culture in a large volume of liquid medium (10 liters or more) and harvesting the cells by centrifugation with the Sharples centrifuge (*2*). Our initial efforts were unsuccessful until we discovered the requirement for a high *p*H by this organism. This report outlines the essential details of the culturing techniques we have adopted for growing and harvesting *M. lysodeikticus*. We expect to publish a more detailed report later.

The organism was obtained from the American type culture collection, No. 4698. The stock culture is maintained on agar slants at room temperature by transfers approximately once every 7 or 8 days. The viability of these organisms is remarkably stable at room temperature. The agar medium consists of the following: 1 percent yeast extract (Anheuser-Busch), 2 percent dextrose, 0.5*M* K_2HPO_4 (8.7 grams per liter), and 1 percent of a salt solution containing the following: 4 percent $MgSO_4 \cdot 7H_2O$, 0.2 percent NaCl, 0.2 percent $FeSO_4 \cdot 7H_2O$, and 0.16 percent $MnSO_4$. The *p*H of the medium is brought up to approximately 8.0 with KOH (1 milliliter of 50-percent KOH per liter) before the 2-percent agar is added. The same medium is also used to grow the inoculum culture for the submerged cultures.

The liquid medium of the submerged cultures is made up from three solutions as follows. (i) Solution A: into a 12-liter flask are placed 9.1 liters of distilled water, 100 milliliters of the aforementioned solution, and 85 grams of $NaHCO_3$ (final molarity in medium 0.1*M*). The surface of the liquid is sprayed with Dow Corning antifoam A spray. The flask is then autoclaved with a cotton plug for 1 hour at 15 pounds pressure. (ii) Solution B: into a 1-liter flask are placed 100 grams of yeast extract and 400 milliliters of water. (iii) Solution C: into a 500-milliliter flask are placed 200 grams of glucose and 300 milliliters of water. The two cotton stoppered flasks are autoclaved for 20 minutes at 15 pounds pressure. After cooling, solutions B and C are added to solution A aseptically.

The inoculum is prepared either from surface cultures or from submerged cultures (with the afore-mentioned liquid medium) grown by the shake flask technique in 250-milliliter erlenmeyer flasks. We prefer the former method because it permits us to detect by visual inspection any possible contaminants in the inoculum. For growing the surface cultures, we have used Corning No. 4422 culture flasks that contain 1 liter of the agar medium. Approximately 48 hours after the agar medium has been inoculated, the cultures are harvested by washing the surface with sterile distilled water. If the cultures are not contaminated in this process, two successive harvests can be made from the same flask. The contents of two flasks are added to one submerged culture flask.

After inoculation, the liquid culture medium is vigorously aerated by sucking air through the liquid. The aeration system consists of an air exhaust and an air intake line which pass through a rubber stopper in the flask. The intake air is filtered through sterile cotton and dispersed in the medium by means of a sintered glass plug. The entire aeration apparatus is autoclaved separately. The cells are harvested from the liquid medium after 48 hours of growth at room temperature by means of the Sharples centrifuge.

The average yields of cells from the submerged cultures have been approximately 3.5 grams (dry weight) per liter of medium. A few experiments have been made with the shake flask technique and on one occasion the yield was 9 grams per liter. The total yield of the latter was small because of the limited number of flasks that could be handled conveniently. We have not yet attempted to determine the reason for the difference in yield by the two methods. However, the two most likely reasons are (i) larger initial inoculations by the shake-flask method and (ii) better agitation of the bacteria in the shake flasks. It was pointed out to us that growth of aerobic organisms in large-scale liquid cultures is more vigorous if the cultures are simultaneously agitated and aerated (*3*). It is of interest to note that either the potassium or the sodium salt of bicarbonate may be used and that the molarity of the buffer can be as high as 0.2*M* without causing any detectable effect on the growth of the organism. However, 0.1*M* bicarbonate is adequate to keep the *p*H above 8.0 for 48 to 72 hours. The high *p*H is reached by the simple procedure of boiling off CO_2 in the autoclaving procedure.

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References and Notes

1. D. Herbert and J. Pinsent, *Biochem. J. London* 43, 193 (1948).
2. I wish to acknowledge the technical assistance of Elizabeth Gaudy in this study. This investigation was supported by research grant C-2550 from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.
3. L. A. Underkofler, private communication.

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Plea for the Extension of Biological Abstractions

In any comparison of the biological and physical sciences one is struck by an apparent dearth of great theoreticians and radical turning points in the former, particularly within the present century. This is not to make the absurd assertion that the biological sciences have not had their great moments, but such moments have usually sprung from what immediately preceded. The progress has been amazing—but staid. There have been no grand explosions, one might almost say, since Pasteur and Darwin. The mathematical sciences understandably lend themselves to the purely speculative. However, there is more than such inherent difference. Those in the physical sciences, although undoubtedly awed by sheer magnitude, dare to ruminate upon the whole. That tremendous abstraction, life, is seldom considered in like manner except by philosophers and theologians, both of whom actually deal with man and his opinions of himself—endeavors generally conceded to be valuable although transcendental and unscientific. The biologist, deeply immersed in lymph, sap, or metabolites, seldom troubles himself with sweeping generalizations. It has been suggested that it is a vestige of the Middle Ages, a dear memento of the egocentric universe, a last refuge for man and his dignity. However, even ignoring the supposed scriptural ban does not appreciably facilitate

the impersonal approach to the meaning and ramifications of life.

In my opinion, an opinion for which a surprising amount of support has been found, the time has come to end this constriction of horizons. Shortly, it may well become imperative to end it. I have particular reference to extraterrestial potentialities. Rocketeers have devoted untold hours of meditation and experimentation to their field, as have physicists, astronomers, and mathematicians. Although it is rather doubtful that astrogation charts will be immediately available, their preparation would be largely a matter of erudite compilation. Biologically, that far horizon is populated only with present-day hippogriffs and afreets, the bug-eyed monsters of science fiction. A more reliable segment of the earth's population should be represented on that frontier. Medicine alone has pushed slightly beyond the boundaries of the commonplace with studies of the effects on life of such influences as increased gravity or acceleration and the obverse, weightlessness. This advance has been timorous. The penetration should be both broader and deeper.

Who is to say that man is the ultimate of ultimates? If he is not, why have we not suffered visitations? Perhaps we have. It would require little imagination to justify a *cordon sanitaire* against such barbarians as us. Such thinking must, for the time being, remain moot, merely idle speculation. More to the point might be a reexamination of the indispensability of the opposable thumb to the development of intelligence. Even more to the point might be a consideration of the necessity of free oxygen to the development of superior forms, particularly since one is faced with known anaerobiosis and the theoretical possibility of a more radical redox. Would earthly temperature ranges be mandatory for even the theoretical silicon-based biochemistry? Should not the pioneer of the perhaps not-too-distant future be prepared, to some degree, not only for the vicissitudes of different time and gravity factors and atmospheres of varying degrees of tenability, but also for the eventuality of alien life, intelligent or otherwise? Should he not be supplied with the conclusions of the educated conjecturer, information of value even if little more than a point of departure? Might not the Martian canal(?) lichen(?) proliferate dangerously if offered warmth, air, and moisture? Or would such abundance prove toxic? What agents might control it, or what might be its metabolic properties?

I suggest that a field of intellectual endeavor, which might be termed "theoretical biologics," is worthy of the most serious consideration. Far from being facetious, I feel that the depth of understanding and the scope of knowledge that are required for effective, intelligent work in this field would preclude even the consideration of it by all but candidates for the doctorate and beyond. Unfortunately, many a recognized and respected Ph.D., by the very nature of his extreme specialization, could not boast of sufficient breadth. Perhaps no man ever could. Perhaps only cooperation and teamwork could effect any reasonable synthesis of ideas.

With the advent of the orbital unmanned satellite, perhaps some action will be taken on behalf of those who will form the crews in the next logical development. Certainly it would be stupid, criminally stupid, as well as wasteful of human life and treasure, to send men into space elegantly equipped with every device conceived by the physical scientists only to lose the entire expedition through the action of some biological agency that might have been anticipated. In the physical realm, as in the biological, eventualities beyond imagination might arise. Such could be borne with resolution. Within the scope of the imaginable, no matter how improbable, there can never be any excuse for the lack of foresight and preparation. To precisely such preparation, both experimentally and abstractly, the biologist might well direct his efforts, for, to borrow an expression, "It's later than you think."

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Dietary Casein Level and B-Factor Deficiencies Produced by Antagonists

The influence of dietary protein and carbohydrate levels on B-factor requirements has been given considerable attention. Among other things, it has been thought (*1, 2*) that the requirements are related to the need for B-factors in various enzyme systems in which it could be shown that they play an important part. In most previous studies, however, the presence of tissue stores of B-factors and their production by the intestinal flora interfered with the experiments. It therefore seemed desirable to re-examine some of the problems with the help of reliable antivitamins, which are now available.

Weanling albino rats from a uniform colony were distributed into matching groups of eight to 16 animals. They were fed highly purified diets that contained dextrose and casein in varying ratios and 10 percent lard, together with cellulose, salts, and liberal amounts of all vitamins (*3*). The casein had been made virtually vitamin-free by a special method of purification. All procedures have been previously described in detail (*2*).

When thiamine-free diets containing 5, 30, or 84 percent casein along with 79, 54, or 0 percent dextrose were used, the rats that received the lowest level of protein died earlier than those that received the higher ones; this is in agreement with the observations that replacement of carbohydrate by protein (*3*) or fats (*4*) has a thiamine-sparing effect. It is usually believed (*5*) that this is the result of the fact that thiamine is chiefly involved in the enzyme systems necessary for the metabolism of carbohydrate rather than the systems necessary for the metabolism of protein and fat.

However, when rats were placed on the same diets and injected daily with 50 micrograms of pyrithiamine (the most potent thiamine antagonist, *6*) they died, on the average, much earlier than those that had not received the antivitamin. Moreover, in contrast to the uninjected groups, those on the lowest protein intake survived significantly longer than those on the higher levels (Fig. 1). These experiments were made twice (with groups of eight and 16 rats), and both times the differences observed were found to be statistically significant.

On the basis of this experiment, it can be concluded that the thiamine requirements of rats increase with increasing dietary protein levels if the rigid exclusion of thiamine from the diet is associated with the use of pyrithiamine, which denies the animals the use of both thiamine stores and that produced by the intestinal flora. If these requirements have any relationship to the role of thiamine in enzyme systems and if pyrithiamine exerts no effect other than that of a thiamine antagonist, one must conclude that thiamine plays an important part not only in carbohydrate metabolism but also in protein metabolism.

It has been demonstrated (*2*) that the riboflavin requirements of rats fed the potent riboflavin antagonist, galactoflavin, and maintained on riboflavin-deficient diets increase with increasing dietary protein levels. This could be concluded from observations on survival rates, body weights, and food consumptions. This and other considerations strongly indicated that protein and riboflavin are mutually limiting factors in metabolism.

Increased pyridoxine requirements with increasing dietary protein levels have been found even when no antagonist was administered (*7*). In our experiments, the animals were fed pyridoxine-deficient diets containing 5, 30, or 74 percent casein and were given daily oral feedings of the pyridoxine antagonist, desoxypyri-