Metabolic Capabilities and Deficiencies of Rickettsiae and the Psittacosis Group

James W. Moulder, Ph.D.[1]

The causative agents of leprosy, the typhus fevers, psittacosis, and trachoma have in common, for the moment at least, the distinction of never having been cultivated in artificial media. In their natural hosts they multiply only intracellularly – this is known for certain for the rickettsiae and the psittacosis agents and is generally assumed for the leprosy bacillus. Such obligate intracellular parasites are unusual not only in that they cannot multiply outside of host cells, but also because they *do* multiply inside other cells. This is a property they share with another exclusive group, the facultative intercellular parasites, such as *Brucella abortus* and *Mycobacterium tuberculosis*. Most free-living organisms can no more grow inside a cell than the obligate intracellular parasites can

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grow outside one. So, in examining the metabolic capabilities and deficiencies of these organisms, we are consciously or unconsciously asking two important questions:

1. Why cannot they grow outside of other cells?
2. Why do they grow so well inside other cells?

The answer to the first question almost certainly lies in the accumulation of mutations that reduce biosynthetic competence, mutations that would be lethal in any environment other than the rich intracellular one. Even when we do not know just which biosynthetic capabilities have disappeared, we can make good guesses as to their general nature and the mechanism of their loss. We are encouraged in our search for metabolic lesions by the hope that the long evolutionary adaptation of microorganisms to intracellular existence may have engendered some unusual and interesting nutritional deficiencies or metabolic inadequacies.

The answer to the second question is not nearly so obvious. Intracellular parasites have evolved special mechanisms that enable them to grow and multiply inside suitable host cells. Such mechanisms could be positive—the acquisition of new metabolic patterns that allow a more efficient exploitation of the intracellular environment;—or they could be negative—the appearance of defense mechanisms that enable preexisting metabolic patterns to function in the face of hostile host influences. But whatever they are, we have no real idea at all as to what their molecular basis might be.

We can make a start at answering these questions by asking another one—on the level of intermediary metabolism: what is the simplest representation of the essential life processes? Food is dissimilated to yield energy and synthetic intermediates, and from them are synthesized the macromolecules of DNA, RNA, and protein, together with other essential cell constituents (Fig. 1). These processes must be basically the same in free-living organisms and intracellular parasites. What we want to know is how evolutionary adaptation to an intracellular mode of life has modified the expression of these basic life processes.

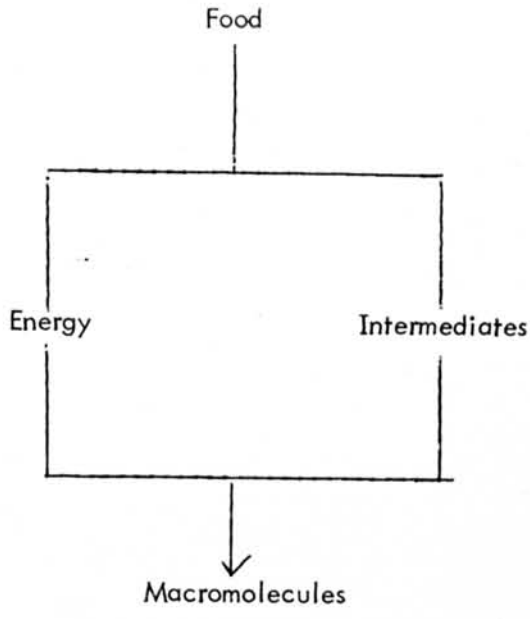

FIG. 1. The simple life.

Study of the metabolism of intracellular parasites is complicated by a factor not encountered in investigating other metabolic systems—the unavoidable intrusion of the host cell, whose rich and varied metabolism often overshadows that of the parasite it harbors. There are two ways of getting around—or trying to get around—these difficulties. First, we can get the parasite out of the host cell in which it has grown, free it as nearly completely as possible from metabolically active host components, and study its enzymatic capabilities *in vitro*. The difficulty here is that we never know for sure that we have left all host activity behind or that we have preserved for *in vitro* observation all of the parasite's *in vivo* enzymatic repertoire. Nevertheless, important discoveries can and have been made in this way; for example, Bovarnick's elucidation of the energy metabolism of typhus rickettsiae ([1,2]). Second, we can make the infected cell our unit of investigation and hope that our ingenuity and a liberal dash of luck will enable us to assess the specific contribution of host and parasite to the overall metabolism of the infected cell. Here the drawback is that we frequently end up with a metabolic equation with two

unknowns and an indeterminate solution, but again this procedure has proven itself an indispensable means for learning about the metabolism of intracellular parasites. It may well be that a goal almost as desirable as that of artificial cultivation is the development of a cell culture system in which the intracellular growth of the leprosy bacillus can be studied under controlled and reproducible conditions.

This is enough generalities; so I shall use the rest of my time to examine three specific topics: energy metabolism, synthesis of proteins, and synthesis of nucleic acids.

Chiefly from the work of Bovarnick ([1,2]) and of Smadel's group (reviewed in ([5])) we have a good picture of the energy metabolism of the rickettsiae (Fig. 2). They do not attack glucose or other carbohydrates, but instead oxidize glutamate via an aerobic mechanism, the tricarboxylic acid cycle, to carbon dioxide and with accompanying oxidative phosphorylation. No energetic advantage is gained by substituting glutamate for glucose, but it is probably significant that glutamate is the most abundant amino acid in the intracellular pool and that its oxidation product is a member of the tricarboxylic acid cycle. The generation of ATP with the energy liberated in glutamate oxidation is a vital activity of rickettsiae. Without a steady supply of ATP, supplied directly or provided by the oxidation of glutamate, rickettsiae quickly perish.

Weiss and his associates ([10,11]) have shown that agents of the psittacosis group attack glucose anaerobically with the formation of pyruvate and the liberation of carbon dioxide. My own group has confirmed their results and has demonstrated the presence in the meningopneumonitis agent of four enzymes of glucose degradation: hexokinase, glucose 6-phosphate de-

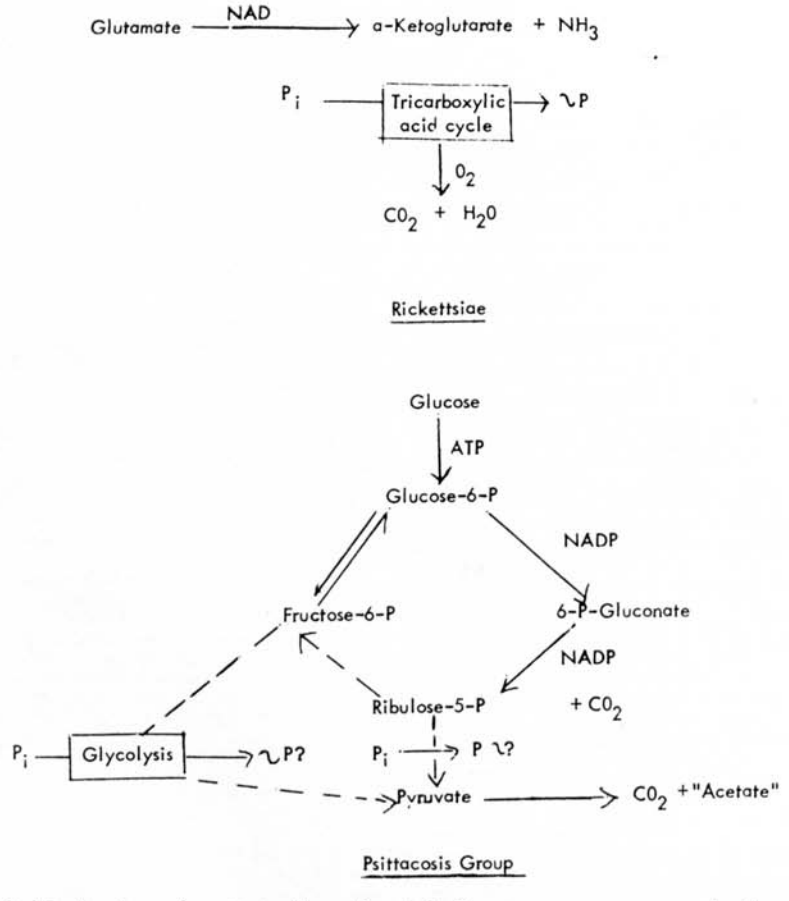

FIG. 2. Mechanisms for generation of metabolic engery—proven and otherwise.

hydrogenase, 6-phosphogluconate dehydrogenase, and phosphohexose isomerase ([6] and unpublished results). These results provide an enzymatic basis for the observed liberation of carbon dioxide from the C1 position of glucose and suggest the presence of a pentose cycle. The series of reactions from hexose to pentose generate no energy. In fact Weiss ([10]) has shown that they require stoichiometric amounts of ATP and NADP for their occurrence. These reactions may, however, be important sources of intermediates: pentose for synthesis of nucleic acids, NADPH for synthesis of fatty acids, and fructose-6-phosphate for synthesis of glucosamine and muramic acid. The production of pyruvate from either pentose or hexose by as yet unknown mechanisms could well generate high-energy phosphate, but this remains to be proven experimentally.

Before leaving energy metabolism, let us make a few comparisons between glutamate oxidation in the rickettsiae and glucose breakdown in the psittacosis group. Both proceed optimally in high K^+, low Na^+ media, media which at least approximate the intracellular inorganic environment. NAD is the first electron acceptor in glutamate oxidation, and the ultimate acceptor is molecular oxygen. NADP is the first known electron acceptor in glucose breakdown. Clearcut evidence of its reoxidation is lacking; although glutathione reductase may be involved. In any event, there is no evidence for aerobic oxidations in the psittacosis group. Glutamate oxidation generates ATP; glucose breakdown requires it. The one generalization we can make is that successful intracellular parasites may exhibit quite different metabolic patterns. Evolution in a common environment, the inside of a cell, does not necessarily lead to the same end result.

Turning now to the synthesis of proteins, I think we can be reasonably certain that the rickettsiae and the psittacosis group agents synthesize their own proteins in ribosomal systems typical of bacteria. Both groups of organisms are susceptible to chloramphenicol and the tetracyclines, antibiotics that inhibit the growth of ordinary bacteria by preventing ribosomal synthesis of protein, and Higashi ([3]) has recently mentioned that his group has actually prepared ribosomes from the meningopneumonitis agent. In my laboratory, Schechter ([8]) has followed the uptake of labeled amino acids into the meningopneumonitis agent growing in L cells and has observed an early and long-continued synthesis of agent protein.

Bovarnick and her associates ([2]) and Bovarnick and Schneider ([1]) have obtained incorporation of amino acids into the protein of isolated rickettsial suspensions (Table 1). The requirements for *in vitro* amino acid incorporation are complex, even more complex than for maintenance of infectivity itself. There is a peculiar double requirement for ATP: for maximum incorporation it must be supplied exogenously and also generated endogenously by oxidation of glutamate. The necessity that amino acids be present in order that one may be incorporated indicates that the rickettsiae have no amino acid pools. Bovarnick and Schneider ([1]) have pointed out that the requirements for protein synthesis in whole rickettsial cells resemble more than anything else the requirements for protein synthesis in subcellular particulates, perhaps because both function inside of cells.

I believe we can be equally certain that the rickettsiae and the psittacosis agents also synthesize their own RNA and DNA by the same general mechanisms used by other organisms. For example, Jones and Paretsky ([4]) have obtained from the agent of Q fever a cell-free system capable of synthesizing RNA.

TABLE 1.—*Requirements for incorporation of amino acids into protein of typhus rickettsiae maintained* in vitro ([1,2]).

Yolk sac protein	Glutathione
	Glutamine
NAD	All naturally occurring amino acids
Coenzyme A	
ATP and other ribonucleotide mono-, di-, and triphosphates	High K^+, low Na^+
	Mg^{++}
	Mn^{++}

In our own laboratory, we have been studying nucleic acid synthesis in L cells infected with the meningopneumonitis agent. Schechter ([8,9]) has used pulse labeling with tritiated cytidine followed by separation of the cell into nuclear, cytoplasmic, and agent fractions. Synthesis of RNA and DNA was first detected 10 to 15 hours after infection and continued at high rates throughout the course of the infection. She used a cytidine label rather than the more conventional thymidine one, because, as first shown by Pelc and Crocker ([7]), thymidine is not incorporated into the DNA (or RNA) of psittacosis group agents. Mrs. I. I. E. Tribby and I (unpublished results) have investigated this remarkable exclusion of thymidine from psittacosis group DNA by adding each of the nine naturally occurring ribo- and deoxyribonucleosides to infected L cell cultures and following their incorporation into meningopneumonitis nucleic acids. All the nucleosides act as precursors of both RNA and DNA in the meningopneumonitis agent except thymidine, deoxycytidine, and deoxyuridine; that is, all except the pyrimidine deoxyribonucleosides. We believe we have an explanation for these curious results. On the basis of work that I cannot describe here in detail, we have concluded that the meningopneumonitis agent can utilize the free pyrimidine bases as nucleic acid precursors. It appears to be impermeable to the pyrimidine deoxynucleosides—and to the nucleotides which the L cell makes from them. The three nucleosides that are unable to act as precursors of agent nucleic acid are those three that are not broken down to free bases by the L cell. These ideas are outlined for the nucleosides of adenine and cytosine in Figure 3. Only deoxycytidine is not cleaved to the free base and only deoxycytidine fails to contribute to meningopneumonitis nucleic acid.

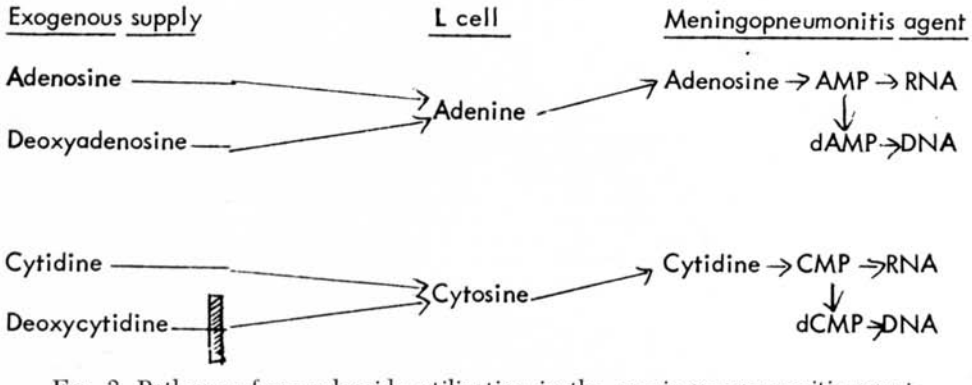

FIG. 3. Pathways for nucleoside utilization in the meningopneumonitis agent.

These experiments are of interest from two points of view. First, they show that the psittacosis group has a more complicated small molecule metabolism than one might have expected. It now appears that they can make a portion of their nucleic acids all the way from the level of nitrogen base and pentose sugar. I had formerly suggested ([5]) that the psittacosis agents might require nucleotide triphosphates for nucleic acid synthesis, an idea that is now clearly wrong. Second, they serve as a warning that in dealing with intracellular parasites, we may encounter unexpected nutritional requirements. Here, the requirement is surprising, not for its complexity, but for its simplicity.

I feel that I am expected to close by making some high level generalizations concerning metabolic capabilities and deficiencies in the rickettsiae and the psittacosis group. I am cautious enough to hesitate—and foolhardy enough to go ahead and do it.

1. Both groups of organisms can generate some metabolic energy themselves, and both, on occasion, directly parasitize the high-energy compounds of their hosts.

2. They make for themselves low molecular weight compounds—like muramic acid—that their hosts cannot supply, and

they depend on their hosts for many others. This dependency reflects in part adaptations to intracellular life and is in part a legacy from their free-living ancestors, whatever they may have been like.

3. They make their own lipids and carbohydrates, proteins and nucleic acids by the same mechanisms as other cells.

4. They have abnormally small pools of oxidizable substrates, amino acids, nucleosides and nucleotides, and oxidation-reduction cofactors, and these compounds pass in and out of these organisms with more than ordinary facility. This makes them extremely vulnerable to irreversible damage when they are transferred from the rich intracellular environment to the poor extracellular one.

5. They are highly adapted both genotypically and phenotypically to intracellular growth. Removal from this environment gives rise to lethal metabolic dislocations.

6. Reproduction *in vitro* of the essential properties of the intracellular milieu will permit artificial cultivation. Delineation of these properties will take much more hard work.

7. Finally, even after we have grown them in artificial media, we shall still be left with the most fascinating question of all, the molecular nature of their successful adaptation to intracellular life. For in our zeal to grow these organisms in artificial media we must never forget that, however successful we may be, they are still going to grow intracellularly in their natural hosts, and that we are still going to have to explain the pathogenesis of their diseases in terms of the consequences of intracellular growth.

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DISCUSSION

Dr. Weiser. Thank you, Dr. Moulder, for those very enlightening remarks. The concepts presented are indeed intriguing and if and when Dr. Moulder succeeds in cultivating various other rickettsiae in an extracellular environment, the results may give us real hope of being able to cultivate *M. leprae*. Dr. Weiss, Deputy Director of the Department of Microbiology at the Naval Medical Research Institute in Bethesda, will open the discussion.

Dr. Weiss. Dr. Moulder analyzed two problems of intracellular parasitism: (1) loss in biochemical competence preventing growth of the microorganism in an extracellular environment, and (2) gain in biochemical competence permitting intracellular growth. Of the two problems the first

is the easier and the only one that I want to discuss. I hope that other discussants will take on the second problem.

In his presentation, as well as in previous publications, Dr. Moulder has made a comprehensive effort to discover similarities among intracellular microorganisms and to draw guidelines for further investigation. I shall act as the devil's advocate and emphasize the dissimilarities.

The typhus rickettsia can well be defined as the prototype intracellular bacterium. It has the main functions of other bacteria, but does nothing well. It produces adenosine triphosphate (ATP) through the oxidation of glutamate, but this ATP is not sufficient for synthesis. Preformed ATP must be added. Nicotinamide adenine dinucleotide (NAD) is the first electron acceptor in glutamate oxidation, but under certain conditions this cofactor can be lost. For optimal stability and function NAD must be present in the medium. Amino acids can be incorporated into proteins, but the rickettsia has no amino acid pool to sustain the reaction, even briefly, under conditions of nutritional deficiency. It has a cell wall, but this wall does not eliminate a requirement for osmotic protection. Nucleic acid synthesis has been studied in *Coxiella burnetii* ([2]) but not in typhus rickettsiae. But even if the rickettsia can synthesize nucleic acids more efficiently than other macromolecules, cultivation in a cell-free medium seems to be a long way off. It needs biochemical assistance over a very wide front. Some of the assistance, undoubtedly, will be easy to provide, such as ATP or NAD, but we must not preclude the possibility that one of the requirements might be the timely inhibition or activation of enzymes, i.e., the mechanism that controls synthesis in other bacteria, as illustrated yesterday by Dr. Goldman. Such a need would be most difficult to satisfy.

This particular portrait of a parasite probably applies only to a small group of microorganisms. As Ormsbee and Peacock ([6]) have recently shown, the close relative of the typhus rickettsia, *C. burnetii*, has a somewhat different type of metabolism. Pyruvate, rather than glutamate, is the chief substrate of intact cells. Preliminary experiments in our laboratory with *C. burnetii* (Weiss and Ormsbee, unpublished experiments) indicated that amino acids are incorporated as in the case of the rickettsia, but the requirements are not so stringent.

If we go still further and consider the psittacosis group of agents, we find a situation that is certainly different (Fig. 1), as pointed out by Moulder. The microorganism utilizes glucose and has at least the three enzyme systems indicated in Figure 1, viz., glucokinase and the two dehydrogenases, plus the other enzymes described by Moulder. It has an absolute requirement for ATP and Mg^{++} and partial requirement for nicotinamide adenine dinucleotide phosphate (NADP). While the dehydrogenase reactions will take place without added NADP, they are markedly stimulated by it. We have reason to believe that pyruvate is produced via a pathway other than pentose. CO_2 is produced from the first carbon of pyruvate. The dissimilation of glucose by this agent is very much like an ideal biochemical reaction: If there is no ATP, no CO_2 is produced; if ATP is added, large amounts of CO_2 evolve. This occurs despite the fact that we are dealing with whole cells. If we start with glucose-6-phosphate, ATP is not required. Pyruvate utilization requires no added cofactors. ATP, NAD, diphosphothiamin, and, probably, coenzyme A, have no effect on the reaction. Surprisingly, lipoic acid has a slight enhancing effect.

A recent experiment by Kiesow and myself (unpublished experiments) illustrates some of the deficiences and capabilities of this agent. This experiment was carried out with the Britton Chance dual wavelength spectrophotometer. With this instrument we can detect very small changes in the state of oxidation-reduction of the pyridine nucleotides of whole cells. If we add pyruvate to a suspension of microorganisms, the reduction of the pyridine nucleotides is rapid and marked. On the other hand, if we add glucose-6-phosphate, which yields approximately the same amount of CO_2, the response is small and irregular. There is a very simple explanation for these observations: the microorganisms have a rela-

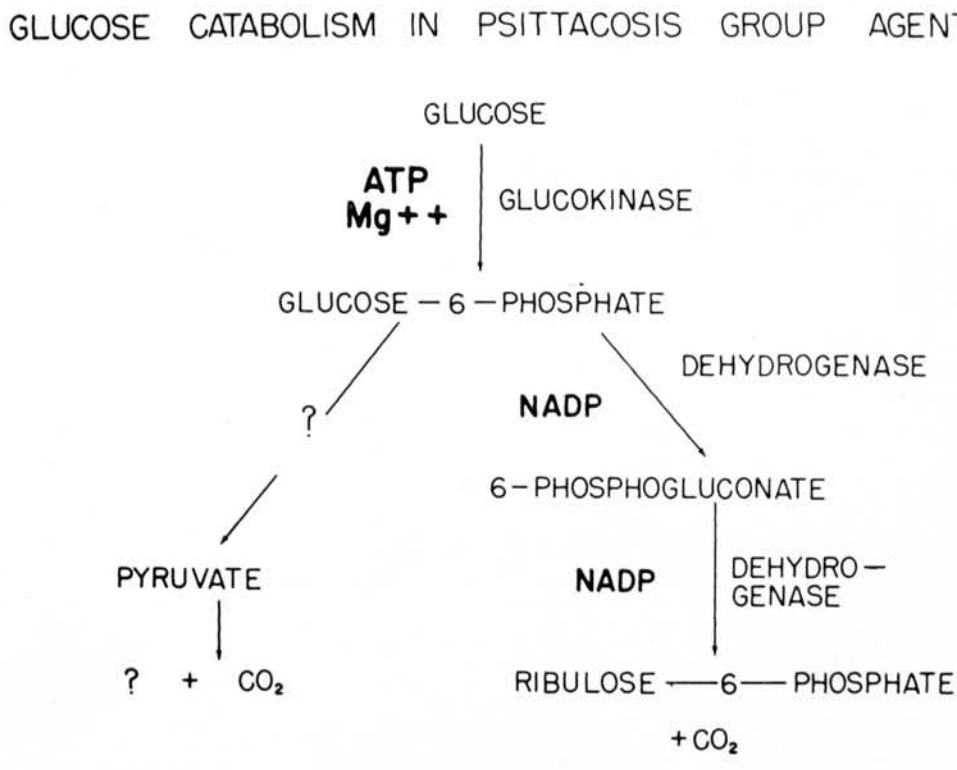

FIG. 1. ATP and Mg^{++} are absolute requirements. NADP is not required, but it greatly enhances the dehydrogenase reactions.

tively large amount of NAD in the oxidized state, which is rapidly reduced when pyruvate is added. Apparently, the microorganism has all the NAD that it needs, since added NAD has no effect on this reaction. It also has a very small amount of NADP in the reduced state. When glucose-6-phosphate is added, reduced NADP is slowly reoxidized, with NAD as the H_2 acceptor. The oxidized NADP is then again reduced in the reaction with glucose-6-phosphate. Since the overall reaction involves a relatively slow and small change in the total state of oxidation-reduction of NAD and NADP, this change is not readily detected. We have thus an example of a microorganism being completely competent to produce and utilize one cofactor and very inefficient in the use of another one that is closely related.

Moulder mentioned the possibility that this agent might produce energy from substrates other than glucose. This hypothesis is subject to investigation. Until the results are known, the alternative possibility can be entertained, viz., that this microorganism produces no energy from substrates and has to be supplied with ATP or phosphorylated compounds. This situation is complicated by the results presented by Moulder, which suggest that the microorganism produces nucleic acids from the nitrogenous bases and can not use some of the nucleotides.

If we put this information together with the data previously obtained in Moulder's laboratory, viz., that this microorganism produces folic acid ([1]) and cell wall components ([3]), even without assistance from the environment, we come to the image of a parasite, which is quite different from that of the rickettsia. Whereas the rickettsia has weaknesses all along the line, psittacosis agents have remarkable strengths as well as remarkable weaknesses.

I wish to mention just one more intracellular microorganism. A few years ago Suitor, in my laboratory ([8]), isolated a rickettsia-like microorganism, which we called *Wolbachia persica* and, which, by

the way, does not resemble a rickettsia. Suitor made very extensive attempts to grow this microorganism in cell-free media and all these attempts have been completely unsuccessful ([7]). However, when we studied the carbohydrate metabolism of this microorganism, it appeared to be extremely active ([9]). An investigation of the lipid metabolism carried out by Neptune and myself ([4, 5]) again yielded excellent results. A few attempts at demonstration of protein synthesis indicated that glucose and other substrates were actively incorporated into protein fractions ([10]). At this point the work was stopped, but it would have been interesting to find out exactly why this microorganism did not grow in any of the media tested. It appears that the metabolic deficiency of this microorganism is a highly specific one and does not involve a broad metabolic function.

When one emphasizes differences among microorganisms rather than similarities, one tends to be pessimistic and, in effect, say that there is very little that we can learn from other systems. However, this is not necessarily so. We are confronted with a number of models of intracellular parasitism. We must study them all and select the one that presents the greatest similarity to the intracellular microorganism we wish to investigate or, even better, to construct our own model from whatever information is available. I hope that those of you who are trying to grow the agent of leprosy will not have to select the model of the rickettsia, which has a series of deficiencies. I hope, instead, that information now available will permit you to select a model of a microorganism that has select deficiencies as well as excellent capabilities. As Moulder has just pointed out—and this seems to be the theme of this conference—a requirement for intracellular growth may surprise you for its complexity or for its simplicity.

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Dr. Weiser. We are now ready for open discussion.

Dr. Dannenberg. I would like to comment on intracellular parasitism in general. First of all the psittacosis group seems to have properties of both viruses and bacteria, and yet *Mycobacterium leprae* looks like a full fledged acid-fast bacterium, although it may also exist in L forms.

Would it be possible, if one considers the lysosomes of macrophages to piece parts of

this conference together? Mycobacteria, when ingested by macrophages, seem to reside in phagocytic vacuoles, or phagosomes. Zanvil, Cohn and Hirsch (*J. Exper. Med.* **118** (1963) 1009), have shown that lysosomal enzymes are poured into the vacuole surrounding such bacteria. As the bacterium divides, the phagosome may divide, and more and more lysosomes may be produced to discharge their enzymes into the new vacuoles. What is the environment in a phagosome? First of all, it is acid. And Dr. Hanks' group have shown that mycobacteria prefer a slightly acid environment. Second, it is full of digestive enzymes, as proteinases, lipases and nucleases. Hanks and Tepper have implied that the lipid coat around acid-fast bacteria tends to impede the absorption of nutrients by the bacteria. The thicker the coat, the more difficulty they have in growing. Could it be that the enzymes in the phagosome digest off this impeding coat and make the intracellular organism better able to absorb nutrients? This may be a reason why it grows in a phagosome. Finally, what are these nutrients? It may be that the digestive enzymes of lysosomes (nucleases, lipases, and proteinases) break down the major building blocks of protoplasm partially but not completely. The resulting peptides, lipid components and short chains of nucleotides may be growth factors for acid-fast bacteria. We have started to purify some of the lysosomal enzymes of macrophages, but will not complete the job for several years. Analogous enzymes, however, could perhaps be used to produce such growth factors by partially breaking down tissue or bacteria elements. The protein, lipid or nucleic acid fragments which result might aid the culture of the leprosy bacillus in the test tube.

Dr. Emmons. It might be interesting to point out that in medical mycology we have intracellular parasites that grow luxuriantly and exuberantly *in vitro* without any special attention to the composition of the culture medium. The three species of *Histoplasma* and *Penicillium marneffei* illustrate this phenomenon. The fungi that we have not succeeded in growing *in vitro* are extracellular. This certainly presents a contrast to the concept that intracellular parasitism is associated invariably with difficulty of *in vitro* culture.

Dr. Segal. I would like to ask Dr. Moulder if the DNA content of these agents has been analyzed as an index of genetic competence, and, secondly, if the base ratios have been analyzed as an index of taxonomic position.

Dr. Moulder. I shall answer Dr. Segal's questions first, and then comment on something Dr. Dannenberg said. First, the DNA content. The DNA content, is about a 5th or a 10th of that of *Escherichia coli*. This would give quite a lot of coding capacity. We have determined the guanine-cytosine ratios of the meningopneumonitis agent by physical methods and it comes out to be about 40 per cent; this is a little disappointing because it is just like that of the host. At the 1965 American Society for Microbiology meetings in Atlantic City, Dr. Gerloff and other investigators from the Rocky Mountain Laboratory gave a very interesting account of some hybridization experiments with meningopneumonitis DNA, and the DNA of three other members of the psittacosis group. There was practically complete homology between meningopneumonitis DNA and each of the other three DNA's tested, a fact indicating, as all of us had thought, that the psittacosis group is a true evolutionary group. They obtained no hybridization between the DNA of meningopneumonitis agent and that of rickettsiae, a number of other bacteria, L cells, etc.

Next, to comment on what Dr. Dannenberg said: First, I do not think you can name one property of the psittacosis group and rickettsiae that is virus-like. I do not believe there is any borderline agent that is really half way between bacteria and viruses. They are either all viruses or all bacteria. I believe the rickettsiae and psitticosis organisms are highly modified bacteria. Now the question of the phagocytic vacuole. I think it becomes very important because the recent fine work of Armstrong and Reed in London and of Higashi in Kyoto, has finally resulted in good electron

micrographs showing all stages from the first absorption of a psittacosis group agent to the host cell membrane and onward. There is no doubt that it is taken in by a process strongly resembling phagocytosis. The invading psittacosis group cell appears in a little vacuole surrounded by a unit membrane, and as the inclusion grows the membrane grows and it seems that the whole life of the psittacosis agent in the infected cell occurs in a vacuole, which is separated from the cytoplasm by a unit membrane. Dr. Dannenberg's discussion of the acidic properties of the phagocytic vacuole reminds me of something I had not thought of before. We have been struck by the fact that the meningopneumonitis agent is quite stable down to pH 5 at which pH it agglutinates, but one can bring the pH back to neutrality and lose no infectivity at all. It will be very interesting to see how far down the pH scale its acid resistance goes. We noticed also many years ago that the psittacosis group agents are resistant to enzymes of all kinds. Therefore, it is entirely conceivable that they could survive in a phagocytic vacuole into which the lysosomal enzymes were being poured.

Dr. Weiser. Our next speaker Dr. P. D'Arcy Hart, was formerly Director of the Tuberculosis Research Unit of the Medical Research Council of Great Britain. He is now associated with Dr. Rees' laboratory at Mill Hill. The topic of his discussion will be "Further analysis of the growth (elongation) phenomenon of *M. lepraemurium in vitro* and relevant studies with *M. leprae*."