Some Aspects of Intracellular Parasitism

Intracellular protozoa enter host cells in subtle ways and escape the cell's digestive processes.

William Trager

Intracellular parasitism represents an extreme of ecological specialization. Nothing less than an intact living host cell will suffice to satisfy the environmental requirements and the nutritional needs of an intracellular parasite. There is a great range of these intracellular organisms, from a relatively large larval *Trichinella*, a worm, in a muscle cell to a small RNA virus in a bacterial cell. In this article I discuss only a small segment of this range, the intracellular parasitic protozoa, with particular reference to the interactions between parasite and host cell at the time of entry of the parasite and during its feeding and growing stages. I will devote some special attention to questions about membranes: whose membranes are they and what do they do?

Entry by Endocytosis

Some intracellular protozoa seem to enter a host cell without any evident activity of their own. Presumably the host cell, upon contact with a parasite, engulfs it by a phagocytic process. In this category are the hemoflagellates of the genus *Leishmania*, whose host-parasite relationships have been extensively studied by Stauber (*1*). These organisms are responsible for a variety of human diseases. The intracellular forms occur in reticuloendothelial cells. They have no organelles visible by either light or electron microscopy that might be supposed to be involved in cell entry.

In the spread of the infection within the mammalian host, it is assumed that heavily infected cells break down and liberate the contained protozoa, some of which are then engulfed by other cells which thus become infected. Why the ingested parasites are not digested, especially when they are engulfed by a macrophage, is a matter of considerable interest. Are they ingested into typical phagocytic vacuoles into which lysosomal enzymes are secreted, and are they somehow resistant to these enzymes? Or are they ingested into a different kind of membrane-bound vacuole, one over which the parasite has some control?

Electron micrographs (*2*) of infected spleen cells suggest the latter. They show that the parasite is separated from its host cytoplasm by two membranes that are mostly very close together but occasionally separated from each other (Fig. 1A). When they are separated the space between them is filled with a finely granular material. The outer membrane, presumably of host cell origin, does not behave like the membrane of a food vacuole. When these parasites divide, each daughter cell becomes surrounded by its individual outer membrane. This host cell membrane controls the flow of materials to the parasite and thus plays a most important role in the parasite's physiology.

Even though the leishmanial parasites do not enter a cell by their own mechanical activity, they clearly interact with the cell in some biochemical way so that the cell forms an appropriate membrane around them—a membrane that, instead of killing and digesting the parasite, proceeds to nourish it.

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Injection into the Host Cell

An altogether different method of entering the host cell is found among the Microsporidia, a large group of intracellular parasitic protozoa best known as parasites of insects and other invertebrates. Pebrine of silkworms, the first infectious disease studied by Pasteur, is caused by a microsporidian *Nosema bombycis*. Besides invertebrates, however, microsporidia are found in fish, where they cause diseases of economic importance, and at least one species parasitizes mammals, producing encephalitis (*3*).

These parasites form resistant spores with a remarkable internal structure (*4*). Coiled within the thick-walled spore is a long fine tubule. When the spore is ingested by a suitable host, or if it is subjected to appropriate conditions in vitro, this tubule is extruded with explosive force (*5*). The sporoplasm contained within the spore is extruded through the tubule and appears at its other end. In recent work, Weidner has studied extrusion occurring in the presence of host cells. Scanning electron microscopy shows a tubule extending from a spore and attached to a host cell. By transmission electron microscopy one can similarly see attachment of the tubule to the host cell and, in addition, the sporoplasm within the cell (Fig. 2) (see *6*). Only a single membrane separates the cytoplasm of the recently injected parasite from host cytoplasm (*7*). We have here a parasite that is not ingested by the host cell but instead injects itself by a special instantaneous method. Whereas the ingested *Leishmania* lies in a membrane-bound vacuole of the host cell the injected *Nosema* lies directly in contact with the cytoplasm.

Motile Invasive Forms

With still other kinds of intracellular protozoa the infective stages are motile and it is assumed that they enter cells through their own mechanical and perhaps also chemical activity. One of the parasites most studied in this respect is *Toxoplasma gondii*. This is a cause of disease in man ranging from lymphadenopathy to retinitis to congenital encephalitis. Until a few years ago *Toxoplasma* was of uncertain taxonomic status, but recent work has shown that it is a coccidian with a typical sexual cycle that occurs in intestinal cells of

cats (*8*). It differs from most other coccidia, however, in that its asexual forms can propagate themselves in a wide variety of cells in many different kinds of animals. But they must have a living host cell; they can grow and multiply only intracellularly. The trophozoites liberated from one infected cell can move about actively for a short time. If they are observed in tissue culture they can be seen to enter new cells. By light microscopy it looks as if they puncture a small hole in the cell membrane and then squeeze through it, as shown in a motion picture by Brommer (*9*). They always enter with the anterior pointed end first, and it is probably significant that this is the end at which special gland-like organelles open (*10*). Perhaps these organelles secrete materials that assist in penetration. Indeed it has been shown that toxoplasmas produce penetration-enhancing substances (*11*).

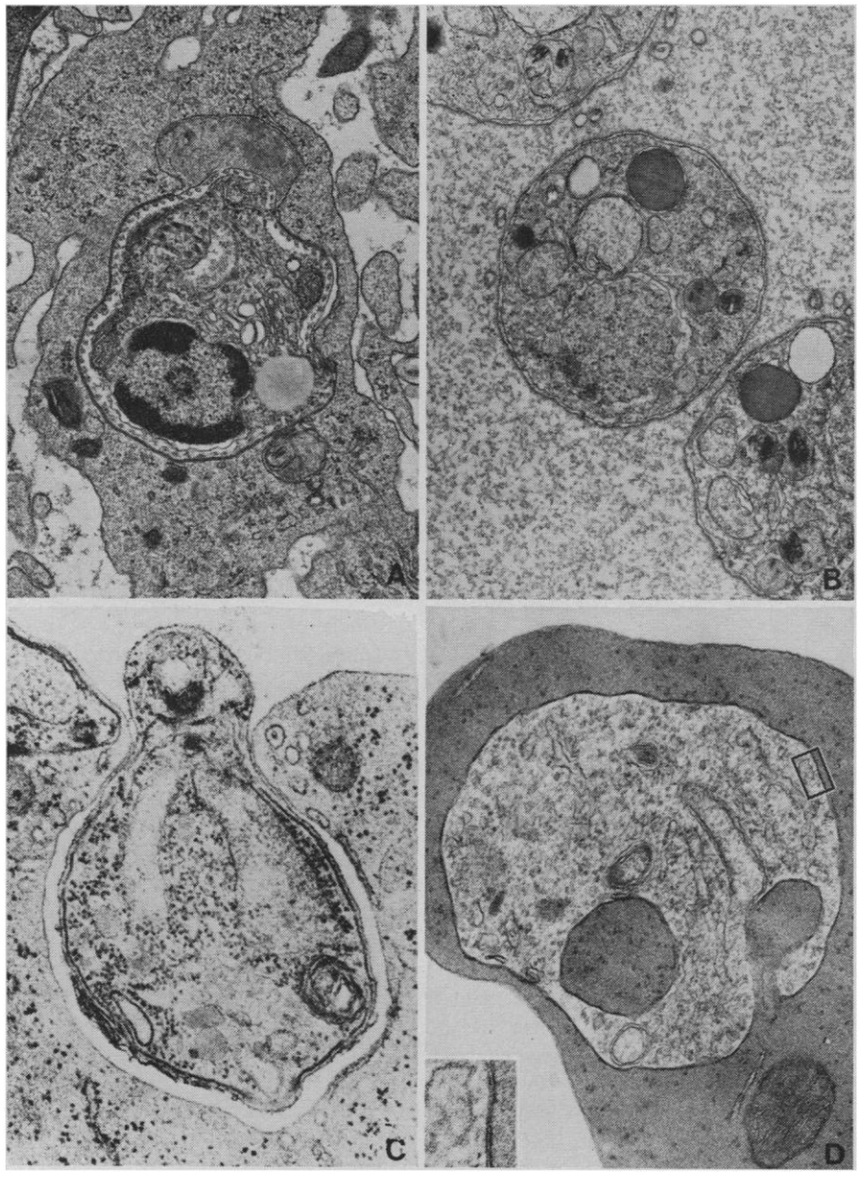

Fig. 1. (A) Amastigote of *Leishmania donovani* in hamster spleen cell (× 19,600). Fine granular material occupies the space where the plasma membrane of the parasite and the surrounding membrane (presumably from the host cell) are separated. [Electron micrograph by S. G. Langreth, Rockefeller University.) (B) *Plasmodium lophurae* after 1 day of extracellular development in vitro (× 14,400). Note the two closely apposed unit membranes bounding the parasite. [Electron micrograph by S. G. Langreth, Rockefeller University] (C) Merozoite of *Plasmodium berghei yoelii* entering a mouse erythrocyte (× 38,900). [Electron micrograph by Ladda *et al.* (*13*), courtesy of the *Journal of Parasitology*] (D) *Babesia microti* within a hamster reticulocyte (× 30,400). The inset shows at high magnification (× 105,000) the single unit membrane separating parasite cytoplasm (on left) from host cytoplasm (on right). [Electron micrograph by M. A. Rudzinska, Rockefeller University]

In recent work, Jones and Hirsch at the Rockefeller University have obtained electron micrographs of thin sections showing invasion of host cells by toxoplasmas (*12*). A very early stage of interaction between a toxoplasma and a macrophage looks just like the beginning of phagocytosis, the major activity of macrophages. But the same thing happens with a toxoplasma interacting with a fibroblast, a nonphagocytic cell. Fibroblasts or HeLa cells will not engulf dead toxoplasmas. Macrophages will, however, pick up dead toxoplasmas, but what they do with them after ingestion is very different from what they do with the live ones. A vacuole containing a dead parasite fuses with lysosomal vacuoles, acid phosphatase and other lysosomal enzymes are secreted, and the dead parasite is soon digested. None of this happens when a live parasite has entered a macrophage. This was clearly shown by first feeding macrophages with Thorotrast to label the food vacuoles into which this material is gathered. The cells were then exposed to both living and dead toxoplasmas, which can be readily distinguished in their fine structure. Vacuoles containing dead toxoplasmas were found fusing with Thorotrast-containing vacuoles. But vacuoles containing live toxoplasmas never fused with the Thorotrast-containing vacuoles and no acid phosphatase was ever secreted into them (Fig. 3). Instead, additional membranes of endoplasmic reticulum were laid down around the vacuole enclosing the parasite (the parasitophorous vacuole). Again, as with *Leishmania*, the host cell reacts in such a way as to nourish the parasite. How the parasite brings this about is among the interesting questions that parasitologists must answer.

In 1956 I was fortunate enough to see a malarial merozoite enter a duck erythrocyte, a process that took about half a minute. The details of what may have been happening during this half minute were not discovered until more than 10 years later when Ladda *et al.* (*13*) published electron micrographs of sections through merozoites apparently in the act of entering an erythrocyte (Fig. 1C). Here again the red cell, which is not a phagocytic cell, never-

theless carries out a process resembling phagocytosis. The parasite, however, has a particular orientation during entry, its anterior going in first. The merozoites of malaria, like trophozoites of *Toxoplasma* and like other members of the Sporozoa, have special organelles leading to the anterior end. These organelles are found in a dividing parasite at the time of merozoite formation and they quickly disappear once the merozoite has invaded a red cell and begun to grow. That they may produce material affecting the red cell membrane is indicated by the work of Herman (*14*). Using synchronous infections of *Plasmodium lophurae* in ducklings he found that extracts of infected blood obtained on the fifth day of the infection, when the parasites are large and are forming many merozoites, increased the osmotic fragility of normal duck erythrocytes. Extracts made on the day before or the day after, when most of the parasites are uninucleate trophozoites, did not have this effect.

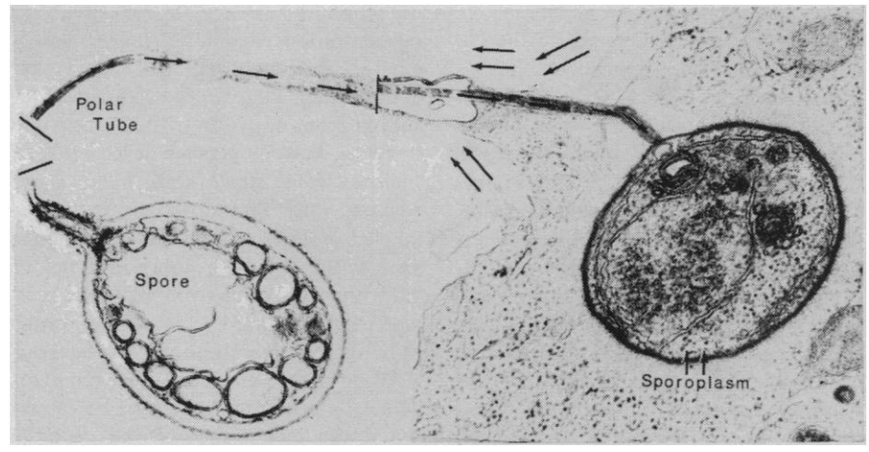

Fig. 2. Discharged spore of *Nosema michaelis* (a parasite of the blue crab *Callinectes*) and newly injected sporoplasm in an EL-4 ascites tumor cell from a mouse of strain C57 BL (× 14,400). Spores primed by exposure to an alkaline buffer, mixed with the ascites tumor cells in tissue culture medium; fixed 5 minutes later and prepared for electron microscopy. The plate is a composite of electron micrographs showing the spore ghost, the hollow discharged polar tube, the penetration of the polar tube into a host cell, with host cell cytoplasm creeping along it (three sets of double arrows), continuity of membrane extending from the polar tube to the sporoplasm, and the sporoplasm with its nucleus. (Preparation by E. Weidner, Louisiana State University.)

Parasite and Host Membranes

Whereas *Toxoplasma* and other coccidia, as shown by Hammond *et al.* (*15*), lie in a clearly defined parasitophorous vacuole, so that host and parasite membrane can generally be readily identified, this is not at all the case with erythrocytic malaria. Here electron micrographs of thin sections show parasite cytoplasm bound by two very closely apposed unit membranes with red cell cytoplasm immediately adjacent to the outer of these membranes (*16*). It is possible that this outer membrane is derived originally from the red cell membrane that invaginates at the time of entrance of the parasite. If so, the membrane becomes greatly altered as it is integrated into the structure and the economy of the parasite. If the parasites are removed from their host erythrocytes by any method they come out with both of their membranes (*17*). These are still firmly attached even after 1 day of extracellular development in vitro (Fig. 1B).

Further indication as to the nonidentity of the outer membrane with red cell membrane can be obtained by immunological methods. Many years ago Stauber and his colleagues devised an excellent serological test for malaria (*18*). Using *P. lophurae*, they prepared, by saponin hemolysis followed by enzyme treatment, suspensions of free parasites. These were readily agglutinated by serum from immune birds that had recovered from infection. We have used similar suspensions of free parasites prepared either by saponin or by immune hemolysis (*19*). Such parasites, as expected, are strongly agglutinated by serum from hyperimmune ducks, serum which has no effect on duck erythrocytes. But what is most interesting is that the free parasites are not at all agglutinated by a potent antiserum to duck erythrocytes. This antiserum is prepared in rabbits by injection of whole washed duck blood cells.

There is, of course, no actual or even theoretical necessity for a host cell membrane around a parasite. This is proved by the increasing number of instances in which no such membrane exists. I have already mentioned the sporoplasm of *Nosema* separated from host cytoplasm only by its own membrane. Another example is provided by the erythrocytic parasite *Babesia* (Fig. 1D (*20*).

Feeding Mechanism and Dependence on Host Adenosine Triphosphate

Whether surrounded by one or by two membranes, erythrocytic parasites feed in the same way, by endocytosis (*16*, *20*). There is increasing evidence that endocytosis is a general method of feeding by protozoa parasitic in other kinds of cells as well as in erythrocytes. In a coccidian, Hammond *et al.* (*15*) have shown endocytic uptake of material from the parasitophorous vacuole. The host cell appears to extrude material into the vacuole and this material seems to be taken up by the parasite. Again, host cell metabolism is modified in such a way as to help nourish the parasite. How this is brought about is another problem faced by parasitologists.

At present, we are trying to find out as much as we can about the nutrition of the parasite, what it requires, what it gets from the host cell, and what it gets from outside the host cell. More has been done along this line with erythrocytic stages of malaria than with any other intracellular protozoon. We know that malaria parasites developing within intact host erythrocytes have relatively few nutritional requirements that have to be satisfied from outside the host cell: glucose, a few amino acids, fatty acids, and a few vitamins (*21*). It seems likely that the amino acids and fatty acids are utilized directly by the parasite but we know that at least one vitamin must first be modified by the host cell. It has been shown that the malaria parasite *P. lophurae* cannot synthesize its own coenzyme A from pantothenic acid and depends on the host cell for this essential cofactor (*22*).

There are indications of an even more intimate kind of dependence on the host cell. Many years ago I found

that extracellular development of *P. lophurae* was much favored by the addition of adenosine triphosphate (ATP) to the medium. The ATP could be replaced by adenosine diphosphate but not by adenosine monophosphate. Hence the ATP was not serving mainly as a purine source. It seemed possible that the ATP might be essential to the functioning of enzymes of active transport on one or both of the membranes separating host and parasite. These considerations suggested investigation of inhibitors of mitochondrial adenosine triphosphatase and of the mitochondrial translocation of adenine nucleotides and of cations. One such inhibitor, the antibiotic bongkrekic acid, was found to inhibit extracellular development of *P. lophurae*.

Bongkrekic acid is itself a most interesting substance. It was originally recognized in cases of food poisoning from a spoiled coconut food product in Indonesia, and was discovered to be a product of a species of *Pseudomonas*. Only recently was it found that bongkrekic acid specifically inhibits the adenine nucleotide translocase as well as energy-linked cation transport in mitochondria (*23*). This material, at concentrations similar to those effective against mitochondria, has effects on extracellular *P. lophurae* like those resulting from omission of ATP from the medium. Fewer parasites develop to the multinucleate stage and, even more striking, only about half as much ^{14}C-labeled proline is incorporated per parasite. With very high concentrations of ATP the inhibition by bongkrekic acid was partly reversed (*24*). These results fit with the hypothesis that exogenous ATP, in nature present in host cell cytoplasm, is required for enzymatic activity at the membranes of the parasite. In this sense the parasite is parasitizing the energy-yielding system of the host. Recently, Weidner and I have obtained evidence for a favoring effect of ATP with an entirely different kind of intracellular parasite. If spores of the microsporidian *Nosema* were caused to extrude into an extract of red cells containing ATP the sporoplasms showed good extracellular survival 4 hours later, as judged by their intact fine structure. In the same medium, but without ATP, the sporoplasms were disintegrating (*25*).

Conclusions

In intracellular parasitism the host cell is a true and hospitable host. The parasite does not have to break in the door. It has subtle ways of inducing the host to open the door and welcome it in. One of the exciting fields in the future of parasitology is to find out what these ways are and why they are sometimes so highly specific that the cell that invites one parasite in will not open the door to another closely related species. Once inside, the parasite not only exploits nutrients already available in the cell, and the cell's energy-yielding system, but it further induces the cell to assist actively in its nutrition. Like a bandit who has cajoled his way in, the parasite now forces his host to prepare a banquet for him. Finally it may destroy its host cell, as in most of the associations I have described herein, or it may stimulate its host cell to abnormal increase in size or to have an altered metabolism with the formation of new products. Or it may even contribute some positive benefit to the host cell or to the multicellular organism of

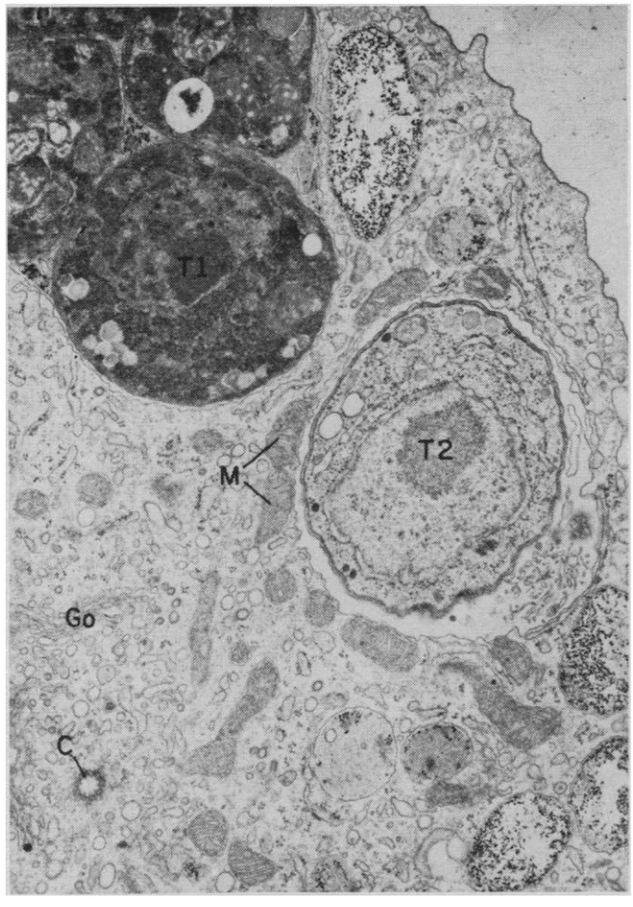

Fig. 3. A macrophage, labeled with Thorotrast, 1 hour after uptake of toxoplasmas. Centriole (*C*) and Golgi region (*Go*) are at lower left; the cell surface is at upper right. The vacuole at upper left contains several dead toxoplasmas (for example, *T1*), which show marked increase in electron opacity and loss of ultrastructural detail. The vacuole contains Thorotrast, and the vacuolar membrane shows no notable association with cytoplasmic organelles. The living toxoplasma (*T2*) shows normal morphology; well defined in this cross section are nucleus and nucleolus, rough endoplasmic reticulum, dense granules, peripheral microtubules, and the inner and outer membranes. The vacuole contains no Thorotrast particles. Microvilli are present in the vacuole at lower right. Host cell mitochondria (*M*) and endoplasmic reticulum are in several places closely apposed to the vacuolar membrane (× 20,000). [Electron micrograph by Jones and Hirsch (*12*), courtesy of *Journal of Experimental Medicine*]

which the cell is a part, so that the two kinds of organisms then live together in a state of mutualism or symbiosis (*26*).

References and Notes

1. L. A. Stauber, in *Immunity to Parasitic Animals*, G. J. Jackson, R. Herman, I. Singer, Eds. (Appleton-Century-Crofts, New York, 1970), vol. 2, pp. 739–765.
2. M. A. Rudzinska, P. A. D'Alesandro, W. Trager, *J. Protozool.* **11**, 166 (1964).
3. R. Lainson, P. C. C. Garnham, R. Killick-Kendrick, R. G. Bird, *Br. Med. J.* **2**, 470 (1964).
4. E. Weidner, *Z. Zellforsch.* **105**, 33 (1970).
5. K. Ohshima, *Parasitology* **29**, 220 (1937).
6. E. Weidner, *Z. Parasitenkd.* **40**, 227 (1973).
7. R. Ishihara, *J. Invertebr. Pathol.* **12**, 245 (1968).
8. J. K. Frenkel, J. P. Dubey, N. L. Miller, *Science* **167**, 893 (1970).
9. W. Brommer, H. H. Heunert, B. Milthaler, *Z. Tropenmed. Parasitol.* **20**, 450 (1969).
10. H. G. Sheffield and M. L. Melton, *J. Parasitol.* **54**, 209 (1968).
11. R. Norby, *Infection and Immunity* **2**, 250 (1970).
12. T. Jones and J. Hirsch, *J. Exp. Med.* **136**, 1173 (1972).
13. R. Ladda, M. Aikawa, H. Sprinz, *J. Parasitol.* **55**, 633 (1969).
14. R. Herman, *ibid.*, p. 626.
15. D. M. Hammond, E. Scholtyseck, B. Chobotor, *J. Protozool.* **14**, 678 (1967).
16. M. A. Rudzinska, *Int. Rev. Cytol.* **25**, 161 (1969).
17. W. Trager, S. G. Langreth, E. G. Platzer, *Proc. Helminthol. Soc. Wash.* **39** (special issue), 220 (1972).
18. L. A. Stauber, H. A. Walker, A. P. Richardson, *J. Infect. Dis.* **89**, 31 (1951).
19. S. G. Langreth and W. Trager, unpublished data.
20. M. A. Rudzinska and W. Trager, *J. Protozool.* **9**, 279 (1962).
21. W. A. Siddiqui, J. V. Schnell, Q. A. Geiman, *Mil. Med.* **134**, 929 (1969).
22. T. P. Bennett and W. Trager, *J. Protozool.* **14**, 214 (1967).
23. P. J. F. Henderson and H. A. Lardy, *J. Biol. Chem.* **245**, 1319 (1970).
24. W. Trager, in *Comparative Biochemistry of Parasites*, H. Van den Bossche, Ed. (Academic Press, New York, 1972), pp. 343–350.
25. E. Weidner and W. Trager, *J. Cell Biol.* **57**, 586 (1973).
26. W. Trager, *Symbiosis* (Van Nostrand Reinhold, New York, 1970), pp. 1–100.

27. This article is based on a lecture given on 17 March 1972 at a symposium at Rutgers University honoring Prof. Leslie A. Stauber on his retirement and is submited in memory of Prof. Stauber who died on 27 March 1973. The illustrations were prepared with the skilled assistance of Erminio Gubert. The work was supported in part by grants from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service (training grant AI 00192 and research grant AI 08989).

Predicting Success in Graduate Education

Improved selection procedures are likely to come from better definitions of "success."

Warren W. Willingham

In recent decades, graduate schools have assumed a major responsibility for the advanced training of a talented segment of American society. Compared with lower levels of schooling, most graduate programs are costly as well as intellectually demanding. Students who complete these programs feed the professions and academic disciplines and constitute a critical national resource. Traditionally, most graduate students have been selected with great care, but until the past decade or so there were relatively few formal statistical studies of that selection process. Such investigations are now common.

There are several possible explanations for the recent interest in predicting success in graduate education. In earlier times, space in graduate schools and the number of applicants were in a rough equilibrium, but burgeoning numbers of applicants in the 1950's and 1960's focused attention on how some were selected and others turned away. These larger numbers of students made it possible to undertake statistical studies in many departments that had previously had too few students to make this type of systematic evaluation worthwhile. Finally, increasing use of selection tests [Graduate Record Examination (GRE) candidates increased from 100,000 to 280,000 per year during the 1960's] suggested the prediction studies with which similar tests are closely associated at the undergraduate level. The purpose of this article is to summarize the results of the substantial number of such studies that have accumulated, to suggest some practical implications for selecting graduate students, and to indicate where further research is needed.

Correlational analysis is the principal research design for evaluating the selection process. One or more predictors (measures of student potential) are evaluated with respect to the extent to which they accurately forecast one or more criteria (measures of student success, typically taken after a year or more in graduate school). The value of a predictor for selecting students varies directly with the size of its correlation with the criterion (*1*). This correlation, called a validity coefficient, ranges from a chance relationship of .00 to a perfect relationship of 1.00, although negative coefficients can occur and perfect validity is not closely approached in practice. Usually more than one predictor is involved (for example, a test and a grade average), and in such cases a statistically weighted composite of the predictors is typically more useful for selection purposes than either predictor alone.

There are a variety of measures that can be used as predictors of success; there are also various measures that can serve as criteria after admission to graduate school. None is entirely satisfactory. Any predictor or criterion should have reasonable construct validity, reliability, and acceptability. By construct validity we mean that the predictor or criterion should be relevant to what we intend to measure. Specifically, it should represent what we want to measure, all we want to measure, and nothing but that which we want to measure (*2*). By reliability we mean that a measure provides a stable estimate from one measuring occasion to another. By acceptability, we mean that a measure is economically feasible, administratively practical, and socially ethical. It is in this context of construct

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