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# Strategies to improve the reliability of a theory: the experiment of bacterial invasion into cultured epithelial cells

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**Abstract**

An analysis is presented of published methods that have been used by experimenters to justify the reliability of the theory of invasion of microorganisms into cultured cells. The results show that, to demonstrate this invasion, many experimenters used two or more methods that were based on independent technical and theoretical principles, and by doing so improved the reliability of the theory. Subsequently I compare this strategy of 'multiple derivability' with other strategies, discussed in the literature in relation to the mesosome, a bacterial organelle that had been detected with the electron microsope, but which appeared later to be an artifact. I propose that different strategies have been applied in this problem, and multiple derivability may have been the decisive one. Finally I discuss the idea that multiple derivability may help to anchor theories in a larger network of theories.


*Keywords:* Multiple Derivability; Robustness; Cell Culture; Invasion of Bacteria; Anchoring of Theories; Experimenter's Regress; Mesosome

## 1. Introduction

When describing the role of experiments in the construction of biomedical knowledge the best one can do is to start with introducing one of the ideas that Ian Hacking

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presented in his seminal book *Representing and intervening* (1983). The idea is based on an experiment that is described by Hacking for the study of the dense bodies that may be observed in blood platelets with the use of an electron microscope at low magnification. To decide whether these dense bodies were artifacts or not, the same platelet samples were studied with a fluorescence microscope. Because in both studies the local arrangement and the structure of the dense bodies was the same, Hacking inferred that the bodies are not artifacts of the electron microscope. Since the two methods of viewing the dense bodies have nothing in common and are different with regard to their physical background, he argued:

> It would be a preposterous coincidence if . . . two completely different physical processes produced identical visual configurations which were, however, artifacts of the physical processes rather than real structures in the cell. (Hacking, 1983, p. 201)

Since Hacking was mainly interested in the reality of entities he could have stopped here. But, although the combination of two different physical methods is an argument that the dense bodies may be real entities, it still does not exclude the possibility that dense bodies are artifacts, although of another type (produced during the collecting, storing and preparation of the blood samples) and neither does it tell us whether the presence of a dense body of blood platelets is something like a reliable theory about their real nature. Hacking is aware of this, because in the next paragraph he writes:

> Note also that no one need have any ideas what the dense bodies *are*. All we know is that there are some structural features of the cell rendered visible by several techniques. Microscopy itself will never tell all about these bodies (if indeed there is anything important to tell). Biochemistry must be called in. (Hacking, 1983, p. 201, emphasis in original)

This is the place where I want to introduce my own analysis of an observation made in a cell culture experiment. In my opinion, when Hacking states that we may want to know 'what the dense bodies *are*' (his emphasis), we are talking about some *theory* of dense bodies. My analysis is concerned with a different type of observation that also leads to a theory and my aim is to analyse the strategies experimenters can use and have used to make this theory more reliable. I am using the word 'theory' here in a local sense, which means that it deals with claims or hypotheses made by inductive inference from observations. Examples of such local theories include a bacterial colony count that makes us infer that bacteria may have invaded cultured cells, and electron microscope pictures that make us infer that mesosomes are subcellular particles in bacteria (I will discuss these examples later). The main strategy I want to discuss is the one hinted at by Hacking when he suggests that biochemistry should be called in to tell us more about dense bodies. Thus, biochemical methods may lead to a new knowledge claim about dense bodies in blood platelets, made by another step of inductive inference.

This type of strategy has been given several names, such as 'robustness' (Wimsatt, 1981; Culp, 1994, 1995), 'triangulation' (Star, 1986) or 'independence of route' (Hudson, 1999). I have adopted the name 'multiple derivability' (Nickels, 1989) because this is the name that represents the dynamic principle of the strategy. Multiple derivability is the strategy by which a theory is supported by the evidence obtained through two or more independent methods that differ in the background knowledge on which they are based.

The aim of my paper is to demonstrate that multiple derivability is an important strategy to improve the reliability of a theory. To do this I will use the case of an experiment with cultured cells and bacteria. This will lead to a proposal for a hierarchical structure of theories and to some speculation about theory networks.

## 2. The invasion experiment

The experiment that will be central to my analysis of scientific theories and hypotheses is a cell culture experiment with bacteria in which I was involved myself as an advisor for the methods of how to obtain cells from bovine udder and how to culture them. The context of the problem was bovine mastitis, a disease caused by infection of a cow's lactating udder by such microorganisms as *Staphylococcus aureus*, *Streptococcus uberis* and *Escherichia coli*. The infection may result in various subclinical or clinical manifestations and since reduced milk production may be the main consequence it can lead to severe economic loss. The experiments were aimed at finding a relation between *E. coli* strains isolated from the milk of inflamed cows and their capacity to invade epithelial cells of udders of normal cows cultured *in vitro*. From epidemiological studies and studies of the dynamics of *E. coli* infections in cows it had been hypothesized that the bacteria may be hiding in local reservoirs inside the animal; the invasion experiments were performed to study the possibility that they hide inside epithelial cells of the udder. The details of the experiments have been published recently (Döpfer, 2000, 2001).

An essential part of the experiments was the determination and quantification of the bacteria that had invaded the cells. Since adhesion of the bacteria to the surface of the cells was evaluated as well, both adhesion and invasion were measured more or less simultaneously in one experiment. The procedure was as follows. Bovine mammary epithelial cells were cultured as monolayers of known surface area dimensions; a suspension of a known concentration of bacteria (*E. coli* in these experiments) was placed on top of the epithelial cells and monolayers plus bacteria were cultured at 37°C. After one hour the monolayers were washed, the cells were lysed and the number of bacteria were counted by serial dilution of the resulting suspensions and plating known volumes on agar plates, on which each bacterium gives rise to one colony. The count was supposed to represent all bacteria that had adhered to and invaded into the cells. In the same experiment a number of different monolayers incubated with bacteria were washed to remove free bacteria, but here extracellular bacteria were killed by incubating them with antibiotics. Subsequently the monolayers were washed again (to remove antibiotics), cells were lysed and

bacteria counted; this count was supposed to represent the bacteria that had invaded the cells. By subtracting the latter from the former the number of adhering bacteria was obtained. In this paper I will only discuss the bacteria that had invaded. As a control for the proper performance of the procedures, strains of two other bacterial species were placed on the cells: a *Salmonella* strain that is known to be very invasive as a positive control and an *E. coli* K12 strain that is known to be non-invasive in all cell types studied so far as a negative control.

When we concentrate on the invasion phenomenon itself one may ask how the experimenter knows that she has demonstrated invasion of the bacteria into the cultured cells. The argument may go as follows: given that, after washing, the count from the culture-well in which the monolayer and the bacteria are present together represents the bacteria that have adhered (and are extracellular) plus the bacteria that have invaded (and are intracellular), then killing the extracellular bacteria in that well results in a count that represents the intracellular bacteria only. In fact, a purely inductive argument is given here because the observation of differences in count leads to a more general hypothesis, or, as I shall call it throughout this paper, the theory of invasion of the bacteria into the cultured cells.

It should be added here that a number of steps have been or could have been introduced into the procedure to make sure that the method as such works well. Simultaneous incubation with *Salmonella*, which has been shown to be invasive, eliminates a false induction that the bacteria under test are non-invasive; simultaneous incubation with an *E. coli* strain, which has been shown to be non-invasive, is used to eliminate a false induction that the bacteria under test are invasive; measuring the colony-forming potential of the bacteria after antibiotic treatment may be a test of the premise in the argument given above that the extracellular bacteria have been killed. However, the theory of bacterial invasion may not be true. The results obtained by colony counting can also be explained by a fraction of the bacteria adhering to the cell surface that has escaped killing by antibiotics because of a very local production of an antibiotic-degrading enzyme by the epithelial cell in the immediate environment of the bacterium. Thus the theory, based on colony count alone, is certainly underdetermined. The theory needs more support and it is here that multiple derivability comes in as a major strategy.

## 3. Bacterial invasion as a reliable theory

The first question to be addressed here is how the experimenter, who performed the invasion experiment described above, is justified in concluding from her results (colony count) that the bacteria have invaded the cells. Given the controls she included in the set of her experiments she may be sure that her methods are reliable, therefore two strategies for justification are open for her: first, she could make a connection between her own experiment and the results that have been obtained by other experimenters who used the same method; second, she could perform a new experiment with the same purpose, i.e. demonstrating invasion, but with a different, independent method.

The first strategy raises no special problems for interpreting the experimental results. By being able to derive support from previous experiments, performed by other experimenters using the same method, our experimenter does not have to justify the inductive argument that her bacterial colony count shows that the bacteria have invaded her cultured cells. By using an accepted method for which this justification has already been made, she makes herself and her experiment and its interpretation part of a network of results, interpretations, and hypotheses that contributes to the reliability of her own interpretation and hypothesis. The invasion experiments described above were published with bacterial colony count as the only method to demonstrate that the bacteria had invaded the cells. However, the method that had been used was indeed based on comparable methods that had been published earlier and that were in use in various laboratories. The authors thereby conformed to the standards of the community of biomedical scientists studying bacterial invasion. Nevertheless, it may be asked how this standard has been constructed.

This leads to the second strategy which is the performance of a new experiment by using a different, independent method. The terms 'different' and 'independent' refer to a type of reasoning in which a second experiment, the results of which can be interpreted to support those of the first experiment, is based on physical processes or theoretical presuppositions that differ from those of the first experiment; as stated above I have adopted the name 'multiple derivability' for this strategy. When we apply multiple derivability to the bacterial invasion experiment it is clear that repeating the experiment with another antibiotic does not help the experimenter to justify her generalization of invasiveness since the processes and theoretical principles of the new method are largely the same. But when she is able to show that the bacteria can be found inside the cultured cells by using electron microscopy (EM) she has improved her inductive reasoning, because EM is a method that uses physical principles instead of biological principles and has a completely different theoretical background.

To see how the strategy of multiple derivability had been applied to the bacterial invasion theory, how past experimenters justified their belief that bacteria had invaded their cultured cells, I performed a retrospective literature research. To this purpose I searched the Medline database, using as keywords the entries 'invasion', 'vitro', and the names of several bacterial species (*Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium*); I searched back to approximately 1980 and then worked further back in time by using references in consulted publications. The results are shown in Table 1. Before discussing them some preliminary remarks should be made.

First, the table is certainly biased, in the sense that only official publications are used as a source. Although I have no information about the unpublished considerations and motivations the experimenters may have had to perform their experiments as they did, I do not consider this as a disadvantage because ultimately the experimenters wanted to bring their conclusions and reasons for holding them into the public domain and have them put to the test by the readers, and the editorial board and the peer reviewers of the journal of their choice.

Second, the table is not complete. Many more publications of bacterial invasion

Table 1
Publications of bacterial invasion in cultured cells

| | Author | Year | Cell or tissue | Microorganism | Invasion method | Justification |
|---|---|---|---|---|---|---|
| 1 | Smyth | 1916 | Chicken embryo | Tubercle bacilli | Giemsa, microscopy (drawings) | Accurate focusing and the location around nucleus |
| 2 | Shepard | 1955 | Hela | Tubercle bacilli | a. Giemsa, microscopy<br>b. Photographs | 1. Bacteria localized around nucleus in a characteristic manner<br>2. Growth not inhibited by factors that inhibit extracellular growth<br>3. Sections of infected cells perpendicular to plane of growth of bacteria showed surrounding by cytoplasma |
| 3 | Cohn *et al.* | 1959 | a. Mouse lymphoblasts<br>b. L cells | Rickettsia tsutsugamushi | Giemsa, microscopy | Characteristic localization around the nucleus |
| 4 | Gerber *et al.* | 1961 | Henle 407 intestinal epithelium | Shigella flexneri | a. Counting of bacterial colonies after lysing cells<br>b. Photographs | Distortion of nucleus |
| 5 | LaBrec *et al.* | 1964 | a. HeLa cells<br>b. Living animals | Shigella virulent and avirulent | a. Giemsa and microscopical photographs<br>b. Fluoresc. antibodies on sections of intestine | |
| 6 | Jones *et al.* | 1972 | a. HeLa cells<br>b. L cells | Toxoplasma gondii | a. Phase contrast microscopy<br>b. EM (only pseudopodia shown in early stage) | |

(*continued on next page*)

Table 1 (*continued*)

| | Author | Year | Cell or tissue | Microorganism | Invasion method | Justification |
|---|---|---|---|---|---|---|
| 7 | Friis | 1972 | L cells | Chlamydia psittaci | a. RA labeled bacteria (dynamic study)<br>b. EM: intracellular morphology<br>c. Giemsa and microscopy: control of uptake | |
| 8 | Kuo *et al.* | 1973 | HeLa cells | Chlamydia spp | Giemsa, microscopy | |
| 9 | Gianella *et al.* | 1973 | HeLa cells | Salmonella typhimurium strains | a. Killing of extracellular bacteria by antibiotics.<br>b. Giemsa and microscopy | All strains tested for invasiveness in rabbit ileal loops and guinea pig conjunctiva |
| 10 | Lycke *et al.* | 1975 | HeLa cells | Toxoplasma gondii | a. Phase contrast<br>b. EM for mechanistic studies | |
| 11 | Byrne | 1976 | L cells | Chlamydia psittaci | a. RA Chlamydia; washing of free bacteria, TCA precip. and RA determ.<br>b. Giemsa and microscopy | |
| 12 | Kihlström *et al.* | 1976 | HeLa cells | Salmonella typhimurium | EM | Previous experiments by the same group |
| 13 | Byrne | 1978 | L cells | Chlamydia psittaci | RA Chlam., washing and trypsin to remove attach Chlam.; remainder = intracellular | Trypsin releases surface-bound Chlamydia |
| 14 | Hale *et al.* | 1979 | Henle intestinal epithelium | Shigella flexneri | a. Giemsa and microscopy<br>b. Fluorescent antibody after methanol<br>c. Counting before and after extracellular killing by kanamycin | Second and third method used to validate the first |

Table 1 (*continued*)

| | Author | Year | Cell or tissue | Microorganism | Invasion method | Justification |
|---|---|---|---|---|---|---|
| 15 | Hale *et al.* | 1979 | Henle intestinal epithelium | Shigella flexneri | EM | EM with thorium to show glycocalix of cell membrane and its absence around microorganism |
| 16 | Gregory *et al.* | 1979 | L cells | Chlamydia psittaci | scanning EM | |
| 17 | Jones *et al.* | 1981 | HeLa cells | Salmonella typhimurium | a. Phase contrast<br>b. Fluorescent antibody before and after methanol | |
| 18 | Bukholm *et al.* | 1982 | HEp-2 cells | Salmonella spp | a. Counting with incident UV and Nomarski in same microsc.<br>b. Scanning EM | |

have been published than are shown here. The publications I have included are those that show how experimenters defended their conclusions in the light of the strategy of multiple derivability. For this reason I concentrated on older literature, because from 1980 onwards more and more publications refer for their methods to work published before.

Third, some of the experiments described in the publications in the table were performed to quantify invasion, not to confirm that invasion was possible. In those cases methods were used to make this quantification feasible, such as marking the bacteria with a radioactive label. The reason that I have included them in the table is because in the same study a second method was used, based on different methodologies and theories, which I considered an example of multiple derivability.

Fourth, with one exception all the publications in the table describe research performed in a period that starts in 1955. This certainly has to do with the introduction of immortalized cell lines into the studies of cell biology in those years. The exception is a publication in 1916 in which cultured embryonal chicken tissues were used (Table 1, row 1). In fact, what was described there was the process of phagocytosis, a process firmly established as a biological theory by Metchnikoff but known since 1847 (for more details of Metchnikoff's work and phagocytosis in particular see Tauber & Chernyak, 1991, Ch. 5, 6). The remarkable aspect of the investigations of Smyth (row 1 of Table 1) was his use of monolayer cells that are attached to the bottom of the culture dish, whereas all other investigators of phagocytosis before and after him used white blood cells (leukocytes), obtained from different species of animals, that stay in suspension during culture. The uptake of bacteria into these cells was seen as an active process performed by the leukocytes, by engulfment by cellular pseudopodia. The demonstration and quantification of phagocytosis was done by making blood smears on glass slides, staining them chemically so as to contrast cells and bacteria, and counting the bacteria taken up by the cells. Possibly because his approach was so different, Smyth may have felt forced to justify his conclusion that he had seen bacteria inside his cultured cells. Another reason may have been that Smyth found *in vitro* what Robert Koch had described in histological sections *viz*. the presence of tubercle bacilli in giant cells (multinucleated clusters of fused cells); since Smyth found the formation of giant cells in his cultures, with tubercle bacilli inside these giant cells, he may have seen this also as a reason to justify his conclusion that his bacteria were inside the cells. The role of phagocytosis research will be discussed again later. It may be useful to remark here that phagocytosis is uptake of bacteria (and other particles) by cells that are especially equipped for that purpose, whereas invasion is the process of entrance of bacteria into other cell types by mechanisms in which the bacteria themselves are actively involved.

When we consider the methods used to study invasion of several types of microorganisms and how the experimenters have tried to convince themselves and their readers that they were justified in concluding that they had observed invasion into cultured cells—that bacterial invasion is a reliable theory—we see (Table 1) that in most of the selected publications two or more different types of methods were used. In addition, some experimenters, while discussing the use of their methods, included arguments that they used those different methods especially for that purpose.

One of the oldest publications I am aware of in which this justification was introduced into the Methods section is shown in row 2 of Table 1. The authors used Giemsa staining of the bacteria and viewed the cells with a light microscope; in their publication they showed some photographs. More importantly, in the Materials and methods section they described some additional experimental approaches by which they tried to justify their conclusion. Next to stating that in the Giemsa staining the bacteria localized around the nucleus 'in a characteristic manner', they reported that the bacteria inside the cells did not stop growing after various treatments (i.e. frequent washing, streptomycin treatment, culture in human serum) which they should have done had they been extracellular. As a third method they made sections of infected cells 'perpendicular to the plane of growth of the bacteria' in which the latter were shown to be surrounded by the cytoplasma of the cells. I have several comments about this. It is not clear how we should interpret the phrase that the bacteria were localized in a characteristic manner; apparently it refers to a type of localization that may be familiar to scientists who also had worked with uptake of tubercle bacilli by cells. Smyth in 1916 (row 1) used the same argument and therefore it may have been a well-established criterion. The third method, although not theoretically independent from the first, is in principle technically independent; however, it is difficult for me to grasp how it has been performed. Finally, the second method is based on an experimental manipulation, which is based on slightly different theoretical principles, i.e. inhibition of growth of bacilli.

Many of the methods used in the subsequent studies are based on light microscopy after Giemsa (or another chemical) staining. In the studies of rows 3 and 4 characteristic localization around the nucleus is again used as an observation statement, but in row 4 the high amount of bacteria distorted the morphology of the nucleus, as shown in photographs, which was accepted as an indication of the bacteria to be present intracellularly. The study of row 4 also used colony counting of lysed cells after longtime incubation to permit intracellular growth and in the meantime washing away extracellular bacteria. Other methods that were used to demonstrate the presence of intracellular bacteria are: phase contrast microscopy for direct counting in living cells (rows 6 and 10); electron microscopy (EM) (rows 7, 10, 12, and 15); use of radioactive (RA) labeled bacteria (rows 7, 11, and 13); killing of extracellular bacteria by antibiotics and subsequent counting of the remaining cell-associated bacteria (rows 9 and 14); use of fluorescent antibodies against bacteria after permeabilization of the cell membrane (rows 14 and 17); and counting all cell-associated bacteria with incident ultraviolet light followed by Nomarski (differential interference contrast) microscopy for detection of extracellular bacteria, both with the same microscope (row 18). The study of row 9 was explicitly designed to investigate whether *in vivo* studies could replace the use of experimental animals.

Three studies warrant further clarification. In the investigations of LaBrec et al. (row 5) two strains of *Shigella flexneri* were compared that differed in virulence as was shown in living animals; by also using them *in vitro* a relation could be established between virulence *in vivo* and invasiveness *in vitro*. The study of Gregory et al. (row 16) used scanning EM as the only method to demonstrate that the microorganisms had invaded. The authors presented some pictures to support this con-

clusion; however, they presupposed an interpretative skill that I expect to be absent in many interested readers (I certainly lacked it). The studies of Hale et al. (rows 14 and 15) are excellent examples of what the authors themselves called the validation of their methods. In the first publication (row 14) the second method—fluorescent staining after permeabilization of the cell membrane—and the third method—killing of extracellular bacteria with kanamycin—were explicitly performed to validate the first—Giemsa staining and light microscopy. In the second publication (row 15) additional observations were made with EM to underline the invasiveness of the bacteria, but here care was taken to make sure that the bacteria were not pseudo-intracellular by staining the glycocalix of the cell membrane with colloidal thorium and showing that glycocalix was absent around the bacteria. By comparing the different methods employed here it is evident that different theoretical principles were involved in each: chemical staining and visible light microscopy in the first, staining by antibodies and fluorescent light microscopy in the second, biological effects of antibiotics and bacterial colony counting in the third, and chemical contrasting and electron microscopy in the last. Considerable multiplicity of derivability is shown here.

Although not as detailed as in the studies of rows 14 and 15, most of the experimenters shown in the table used at least two methods to support their conclusions; examples are colony counting combined with photographs (row 4), radioactive bacteria combined with EM and light microscopy (row 7), phase contrast microscopy with fluorescent antibodies (row 17), and so on. Only a few consider one method sufficient. Many refer to studies published before in which the methods had been published by members of the same research group and which are then used without further discussion; I have included one example of this type in the table (row 12).

Some attention should be given to the study of row 1 that I mentioned above. The author justifies the invasiveness of the tubercle bacilli in his tissue culture experiment by remarking that '. . . [i]n all these cases careful focussing showed that the bacilli were within and not on the cells and they usually had a tendency to avoid the nucleus' (Smyth, 1916, p. 287). Although it may be considered an insufficient justification in the light of the strategy of multiple derivability that I tried to show above, it should be noted that the author worked in the context of phagocytosis research and his colleagues may have found his conclusions satisfying because it conformed to the standards of the discipline at that time.

The pattern that emerges here is that biomedical scientists who are presenting a new biological phenomenon, i.e. invasion of a microorganism into cultured cells, generally support their conclusion that their experiments have shown this phenomenon to occur by using at least two methods that are independent with regard to the theoretical and methodological principles on which they are based. It may be concluded that for the establishment of bacterial invasion into cultured cells as a reliable theory multiple derivability has been an important type of reasoning.

## 4. Multiple derivability and other strategies of argument: the mesosome revisited

I now want to discuss my conclusions of the strategy of multiple derivability, as used in the theory of bacterial invasion, in the light of the views that have been offered on a similar problem, that of the mesosome.

The problem of the mesosome has been used to discuss and clarify the process by which scientists go from observations derived from an experiment to a theory inferred from those observations. Again, I use the word 'theory' in the local sense as explained in the introductory paragraph. The mesosome was considered to be a cytoplasmic organelle in bacteria that could be observed with the electron microscope until it was rejected as an artifact that had nothing to do with a living bacterium and was caused by fixation procedures. Different authors have tried to reveal the epistemological reasoning and strategies that were used in the rise and fall of the mesosome.

The discussion was opened by Rasmussen (1993). He described how electron microscopists tried to 'calibrate' their methods by following different fixation and preparation protocols and comparing their results with those of previously applied protocols. In the end he concluded that the scientists involved in the mesosome investigations used several epistemological principles: validation of instrument by theory, calibration with precedent, and calibration with independent methods, strategies I have shown to be applied in the bacterial invasion investigations. In addition, Rasmussen (1993, p. 260) mentioned three more principles: practicality, aesthetics, and inference to function, but I will not discuss these further. In his discussion, and in the discussion of a more recent paper about the same problem (Rasmussen, 2001), he concentrated on showing that the outcome of the process to gain knowledge from experiments is dependent on subtleties and heterogeneities in methods and results, continuous subtle changes in the epistemological principles applied, and on negotiations within research groups.

Rasmussen did not seem particularly interested in the fall of the mesosome ('. . . the purpose of this paper is simply to show the limits of formalizable validation in the establishment of experimental fact' (Rasmussen, 1993, p. 255)) but in both papers he nevertheless gave an indication where to look for it: in his view, an important factor that contributed to the fall of the mesosome was the lack of support from biochemistry. Had biochemists produced evidence that the mesosome is 'the biochemical locus' of one or more activities, the electron microscopists would have felt confirmed in their belief that the mesosome is a real entity. In fact, biochemistry was seen as an important supporting discipline by electron microscopists right from when intracellular structures were first detected by electron microscopes in cells in the 1950s (A. Glauert, personal communication).

Rasmussen (1995) described a case comparable to that of the mesosome in an interesting study of the electron microscopical reality of the mitochondrion. In the competition between two groups that contested for the right interpretation of EM pictures of mitochondria

> . . . Porter and Palade [the winners of the dispute] used the electron microscope to map biochemical reactions onto the spatial representations given by electron micrographs. But claims about biochemical events and entities they were mapping were not generated . . . by the electron microscope; that work of biochemical discovery, characterization and testing were done strictly by biochemists using cell fractions isolated by biochemists.

On the other hand,

> . . . Sjöstrand [the loser] . . . used the electron microscope to establish and test biochemical facts . . . and he interpreted his micrographs according to criteria fully available to the electron microscopist . . . (Rasmussen, 1995, p. 418–419)

Here, support by biochemistry as methodologically and theoretically independent may have been essential (although Rasmussen in a footnote to the above cited paragraphs has doubts about this). In the case of the mesosome, this support was absent and apparently the nature of the mesosome could not be derived from theories and methods other than those of the electron microscope, which turned out to be insufficient.

The discussion of the epistemology of the mesosome was continued by Culp (1994). She was interested in applying the concept of robustness. This concept was originally defined by Wimsatt (1981) as the 'multiple connectedness within a theoretical structure and (through experimental procedures) to observational results'. Robustness is also related to the multiple derivation principle of Nickels (1989):

> A single "proof" is nice, but multiple derivations are naturally preferred in science because they are robust. They amount to what we might term *theoretical replication*—the replication of a theoretical result—by "locking in" the claim as an unproblematic node of the accepted body of theory and practice. . . . [I]f very different theoretical models or research techniques yield the same result, we have robustness. (Nickels, 1989, p. 308; emphasis in original)

Thus, robustness should be seen as the result of multiple derivability.

The mesosome problem was used by Culp to show that differences in robustness may be possible. She argued that the body of data that bacterial cells contain mesosomes was less robust than the body of data that bacterial cells do not contain mesosomes. I agree with her that initially in the study of the mesosome the differences in fixation techniques used by the electron microscopists varied to a degree that made it possible to conclude that robustness was playing a role.

Then freeze-fracture techniques were developed. The advantage of these techniques is that the fixation of the tissues is not dependent on chemical compounds that may introduce artifacts and that the freezing of the tissue fixates it in a far less modified state. Applying freeze-fracture failed to demonstrate mesosomes. In addition biochemical studies were unable to show that mesosomes were entities with special characteristics related to certain functions. Culp then argued that the set of

data (including those of biochemists) supporting the mesosome stopped growing, while the set of data that could reject the mesosome got larger. She argued further that the techniques supporting the mesosome, i.e. the chemical fixation techniques, had a lower degree of theoretical independence than the rejecting techniques. Although I fully agree I also think that the issues start to become mixed up, so I will try to disentangle them a little bit.

The various chemical fixation techniques had a low degree of theoretical independence, but it was sufficient to warrant the conclusion that mesosomes were true intra-bacterial structures; this conclusion therefore had a certain robustness. Then came freeze-fracture experiments. They failed to demonstrate mesosomes and most electron microscopists considered this a refutation of the mesosome as an intra-bacterial structure. But from the perspective of robustness this failure can also be seen as a lack of support for the mesosome: chemical fixation and fixation by freezing did not build up robustness. Now biochemistry comes in. Biochemists were not interested in solving the problem of whether mesosomes were artifacts or not, they wanted to show their functional properties as related to their biochemical composition. Thus, freeze-fracture is a method theoretically independent of chemical fixation, but *within* the domain of electron microscopy as a whole, whereas biochemistry is theoretically independent of electron microscopy as a whole, *outside*. As I see it, multiple derivability takes place at the level of the latter and therefore I propose to call what happened at the level of fixation by chemicals versus fixation by freezing ‘variation in independent methods’. Both, multiple derivability and variation in independent methods produce robustness but on different levels.

The difference between multiple derivability and variation in independent methods can be deduced from Culp’s own description of the case of different methods for determining nucleotide sequences of DNA (Culp, 1995). Culp showed convincingly, more so than in her analysis of the mesosome problem, that the methods had a certain theoretical background independence and that a number of their theoretical presuppositions were sufficiently different to make it improbable that their results were mere coincidence. Yet it is possible to interpret the results as an example of method calibration aimed at establishing a certain nucleotide sequence of a fragment of DNA. Apparently unaware of its importance for the study of experimental reasoning, Culp describes how one of the methods was ‘calibrated’ by comparing its results against the known amino acid sequence of the protein for which the DNA fragment is coding. In her words the event of the comparison went as follows:

> On a Sunday afternoon in late September Sutcliffe and Gilbert [the nucleotide investigators] drove to Knowles’s [who knew the amino acid sequence] house for tea. For the first time, the DNA sequence for the *amp-r* gene and the amino acid sequence of its penicillinase protein product were compared. . . . They began by reading the amino acid sequence and checking the DNA sequence against it. But soon they were using the DNA sequence to order the peptides and to correct a few errors in the amino acid sequence. (Culp, 1995, p. 446)

One of the reasons for reversing the order of calibration may have been that it is

easier to use nucleotide sequences to order amino acids than vice versa, because each nucleotide triplet codes for only one amino acid, but a single amino acid sequence may be coded for by more than one triplet. But it also shows the strategy of multiple derivability at work: both methods, amino acid sequence analysis and nucleotide sequence analysis, are methodologically and technically more different than the two DNA sequence analyzing techniques, and they support each other on the level of the theory of the molecular structure of the penicillinase molecule.

Here I want to go back to the quotations from Hacking that I used in the introductory paragraph. Hacking distinguished two methods for viewing dense bodies in blood platelets that differed in their background theories. Hacking concluded that this decided against dense bodies as artifacts of physical processes. But in addition he argued that we do not know what dense bodies *are* and biochemistry has to be called in for that. This is exactly the distinction between variation in independent methods and multiple derivability that I gave above. Multiple derivability may tell us something about the possible nature of our observations, variation in independent methods cannot do this.

The third participant in the discussion of the mesosome is Hudson (1999), who re-evaluated Culp's arguments and, in rejecting them, came to propose that the fall of the mesosome was caused by a strategy that he calls 'reliable process reasoning'. Contrary to robustness reasoning (called 'independence of route' by him) or background independence, as proposed by Culp (1994), reliable process reasoning has as one of its main features 'the assumption-dependence' of experimental conclusions, the issue of the reliability of the experimental methods that generated the relevant results. In this process of reasoning, the methods are critically re-evaluated in the light of other related findings and the reliability of the results (i.e. the observation of mesosomes) is reassessed; this would lead to the conclusion that freeze-fracture experiments are more accurate and that mesosomes do not exist. In my view, this is the type of reasoning—re-evaluation and reassessment—that took place within the cluster of chemical fixation techniques or could have taken place within the cluster of freezing techniques. The end result of such reasoning may be that a new, independent method could be tried in the same way that chemical fixation was replaced by fixation by freezing. But what reason could experimenters have had to conclude that such reliable process reasoning was necessary? For, suppose that there was no doubt about the existence of mesosomes, i.e. that there was no reason to suspect that mesosomes were fixation artifacts; experimenters would then have found no reason to reassess their methods. Therefore, reliable process reasoning is a process that is used when the theory is under construction or under attack. The publications that Hudson chose to explain how reliable process reasoning might work are those that were published *after* doubt had been raised about the artifactual nature of mesosomes. Reliable process reasoning was not the cause of the fall of the mesosome, it was the consequence of efforts to prevent that fall. However, reliable process reasoning may also take place when a theory is under construction. In the early stages when methods are not yet optimally developed and experimenters are not fully convinced that their results are sufficiently reliable, research groups devote a lot of

discussion to how to improve the quality of their results. This type of reasoning may have more or less similar forms to those that Hudson discusses.

## 5. A hierarchical model of making theories

I want to introduce here a hierarchical model of how local theories are made that is able to include more harmoniously the different strategies described above. I again use the bacterial invasion experiment and I start by imagining that a new student comes to work on bacterial invasion in my research group in order to complete his biomedical research training. He has to start by acquiring the skill of cell culture with bacteria, using the antibiotics protocol for killing extracellular bacteria. We train him in preparing buffers and culture media, in culturing cells and bacteria, in plating bacteria on agar dishes, and so on. The first experiments of students rarely yield results because of their lack of skill: either they are not able to find invasion, or they find too much invasion, or their controls are wrong, and so on. Nobody is worried when the student does not detect invasion and he is asked to ignore his results. Until he has shown sufficient skills, i.e. until he has reliably shown invasion, his results will not be taken seriously. But now suppose that after several efforts he has still not shown invasion. No one in the research group will worry about the theory; the worry will be about the student and about the methods: were the culture media properly prepared? Did the student check the pH of his buffers? What was the quality of the epithelial cells when brought into culture? Were the cells really lysed after killing the bacteria and how did he check that? Here the strategy of reliable process reasoning is at work; it is employed here because observations that belong to the core of the theory cannot be reproduced; the strategy will be used to an even greater extent when the survival of the theory is at stake. Each part of the methodological procedure is discussed to detect where the failure may be located. When this does not lead to solving the problem the student will try to do an invasion experiment with a new batch of cells, or another strain of bacteria, or another type of epithelial cells, leading to a variation in the approach comparable to the use of fixation by freezing as an alternative to the chemical fixation protocols in the case of the mesosomes. The variations are not so large as to make the result a complete new type of observation or a new theory but large enough to make it independent to a certain degree. This is the strategy on which Culp concentrated and which she called robustness.

Now my research group decides to introduce a new approach to solve the uneasiness about the invasion problem: the student is advised (the better students think of this themselves) to go to the electron microscopy department with his putatively invaded cells, to investigate whether the bacteria can be demonstrated inside the cells with the electron microscope, a theoretically and technically totally independent method. This is the strategy of multiple derivability I have described. It is of course possible to describe several alternative outcomes for our imaginary student and the research group. Suppose for example that research groups worldwide never tried EM or any independent method before. When the student in this situation is not able to

show invasion of bacteria by this method either, the group may lose interest in the problem; resolving the problem is not worth all the time and energy. This is what may have contributed to the fall of the mesosome as well.

In my opinion the three methods of theory construction I have described so far, i.e. reliable process reasoning, variation of independent methods, and multiple derivability, form a hierarchical network. It contains three levels.

The first level is the replication of the experiment to check whether it has been performed in the right way if the experimenter has the feeling that the results cannot be trusted. This is the level at which systematic errors in the protocol are discussed and the possibility of methodological artifacts in the results must be considered; it may lead to adjustments in the protocol. This is the level of reliable process reasoning. In the studies of bacterial invasion it may lead to such adjustments as variation in incubation times of bacteria and longer cultivation times of mammary gland cells before bacteria are added.

The second level in the model is the level at which the main body of theoretical background is kept intact but small nodes in it are modified to obtain results that may be considered independent with respect to the methods employed. In the bacterial invasion studies, examples may be the use of cultured mammary gland cells of another cow or of a new isolate of bacteria made from a different source of milk. In microscopy studies, including electron microscopy, an example may be fixation by freezing as an alternative to fixation by chemicals. This is the level of variation of independent methods, a name that I introduced to emphasize that it should be distinguished from reliable process reasoning.

The third level in the hierarchical model is that of multiple derivability, in which two or more theoretically and methodologically different methods enable the experimenter to make observations that are used to infer the same local theory. Each of the independent approaches on the level of multiple derivability contains a number of methodologically divergent methods and in each of those methods reliable process reasoning is a central activity as long as the theory does not have an established form. At each level, robustness is the result of a dynamical process of reasoning and theory making.

For the moment I consider multiple derivability the strongest strategy for local theories such as bacterial invasion and mesosomes. This is shown by the mesosome theory; it collapsed because support for it was absent at two levels: at the level of variation of independent methods (chemical fixation was not supported by fixation by freezing) and at the level of multiple derivability (observations in electron microscopy were not supported by observations in biochemistry). Had biochemistry provided observations from which the presence of mesosomes in bacteria could have been inferred, it might have overruled the lack of freezing techniques; had freezing techniques made the putative mesosomes visible but biochemistry failed to give support to their presence, the final outcome may again have been the fall of the mesosome, but it probably would not have fallen so easily.

If the research group of which our unfortunate student was a part had already demonstrated bacterial invasion by electron microscopy, or by confocal laser microscopy after fluorescent labeling of bacteria, his failure to detect bacterial

invasion by colony counting would not have been able to discredit the invasion theory.

## 6. Anchoring of theories

Nickels (1989), in the passage I quoted above, used the term 'locking in' as a description of how theoretical results may be added in an unproblematic way to bodies of existing theory. This locking in, or 'anchoring' as I prefer to call it, occurs in several domains.

The first domain in which anchoring occurs is the structural domain of a theory; I see this as *epistemological* anchoring. In the previous section I proposed that theories may have a hierarchical structure and this structure, as I sketched it, may be seen as vertical. However, theories can also be seen as horizontally structured. This horizontal structuring takes place when the theories are multiple derived, for instance when bacterial invasion as an electron microscopy-based local theory is combined with, or locked into, a cell biological theory based on colony counts of bacteria and with a microbiological theory based on immunofluorescent detection of the bacteria. Such a local theory of bacterial invasion may initiate new methods, e.g. to diagnose cows sensitive for infection by testing the invasion of bacteria in the cells cultured from udder biopsies of these cows; or it may lead to a research project in which the mechanism of invasion of bacteria is studied in order to find therapies for preventing invasion. In this way a local theory may become part of a larger set of theories, related to both the pathogenesis and the therapy of the disease bovine mastitis; both may be anchored in the body of knowledge made in the field of the epidemiology of mastitis, and the latter anchored into the former as well, leading to a structure of knowledge that I have described earlier (Nederbragt, 2000). In addition, the knowledge of electron microscopy, and of the culture of epithelial cells and of the growth and behavior of bacteria *in vitro*, are based on complexes of theories that are anchored in bodies of knowledge of other, different disciplines. It is tempting to see theories as nodes in a three-dimensional network; the more a theory is anchored in the network the better it will survive. Multiple derivability is the mechanism of anchoring theories in such a network. Apparently, the mesosome was not well anchored.

Both Rasmussen (1993) and Culp (1995) discussed, in the light of their results, the problem of the experimenter's regress. This problem was raised by Collins (1992) when discussing the gravity wave detector. You need a good gravity wave detector to detect gravity waves, but to know that you have a good gravity wave detector you need to be able to observe gravity waves. Collins argued that the problem of experimenter's regress is solved by using criteria outside the problem itself, criteria that are of social nature, such as the personality of the experimenter, his style of presentation, the prestige of his research group, and the authority of his opponents.

Rasmussen (1993) mentioned the problem only casually, stating that the mesosome problem resembles a problem of experimenter's regress because the 'logical circularity entailed in the evaluation of experiments according to their results and in the

way the issue's resolution involves a complex change in the "network" linking the community of scientists together' (Rasmussen, 1993, p. 256). The gist of his argument seems to be that Collins is right and that the problem was solved by social criteria.

Culp (1995) was not so sure. She agreed that the experimenter's regress is a serious problem but she wished to demonstrate that this problem can be solved—that the circularity can be broken—by experimental results alone. She discussed, as I described above, the two methods for DNA sequencing and she argued that robustness can be used for breaking the circularity. I consider this an attractive idea; using strategies of multiple derivability one method for observations, which enables inference to a theory, can be locked into another method for observations, leading to the same theory, saving the theory from circularity. However, in Culp's example the DNA sequencing methods were saved from experimenter's regress by amino acid analysis as well.

I am not convinced that the mesosome problem is a good example of the experimenter's regress. After all, the detection of mesosomes was not necessary to establish that electron microscopy and osmium fixation are reliable methods. Nevertheless, I felt sufficiently intrigued to apply the problem of experimenter's regress to bacterial invasion. For, if bacterial invasion was only a theoretical possibility, if no other method for its study had been available than the colony counting technique, how then could we have been sure that bacterial invasion was a good theory? To me the answer was clear from the outset: apply electron microscopy (or any other technique that is in Table 1). Therefore I tried to find the oldest publications that described bacterial invasion, to see whether any trace of the experimenter's regress could be found in them and, if so, how the problem was solved. As far as I am aware, multiple derivability was the main strategy to circumvent the regress, but even when this was not or not convincingly applied, the experimenter's regress did not turn up.

I have two explanations for this. The first is that Collins is right, that the anchoring of theories occurs in a social context; this is invisible in the official literature because the negotiation of the results is performed using criteria outside the test. Rasmussen (1995) hints at this when he describes that Sjöstrand, the loser in the mitochondrion controversy, lacked the social skills for making biochemists work for his theory. The social domain is the second of the domains in which anchoring of theories may occur. Note that in the example of the mitochondrial debate given above epistemological and social anchoring coincide.

I want to propose a third domain in which anchoring of theories may take place, *viz*. the historical domain, which provides a second explanation for the absence of the experimenter's regress in my data. In my opinion, the bacterial invasion theory was anchored in the much older phagocytosis theory. Experimenters doing phagocytosis research traditionally used white blood cells taken from normal individuals or patients; as a standard method the cells were incubated with bacteria and a smear would be made for Giemsa staining. Rows 2 and 3 of the table suggest that the bacterial invasion literature, as I selected it, was seen as a continuation of this phagocytosis tradition: phagoytosis is explicitly mentioned in the title of the publication of row 2 and in the experiments of row 3 lymphoblasts—a type of white blood cells

of tumorous origin growing in suspension—were used next to L-cells which, in culture, are grown as a monolayer. In addition, the authors of the experiment of row 13 conclude that their results completely conform to the criteria of phagocytosis. Also the intracellular presence of Toxoplasma parasites (row 6) was considered a phenomenon already justified because it had been established much earlier that Toxoplasma is an obligatory intracellular organism (Sabin & Olitsky, 1936). By using a form of multiple derivability the experimenters mentioned here anchored the theory of the intracellular presence of their microorganisms in the existing theory of phagocytosis, thereby avoiding circularity in the evaluation of experiments in which cells in monolayer cultures were used. Later the content of the theory changed from phagocytosis to bacterial invasion but historical anchoring, which with hindsight coincides with epistemological anchoring, had already taken place.

In conclusion, I have described multiple derivability as a strategy to anchor a theory in an existing body of theories. I have also discussed how, in developing theories in their early phases as well as when the theory may be under attack, strategies such as reliable process reasoning and variation in independence of methods are employed. The epistemological anchoring, which is concerned with giving the theory a structure and anchoring it into a network of theories, may coincide with historical and social anchoring of the theory.

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