A Brief History of Phagocytosis

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Abstract

This chapter outlines some of the more significant steps in our understanding of the phenomenon and mechanism of phagocytosis. These are mainly historical, ranging from near the advent of microscopy in the seventeenth and eighteenth century up to the period before the Second World War (1930s). During this time, science itself moved from being the domain of the wealthy enthusiast to the professional and funded university scientist. Not surprisingly progress was slow of the first two centuries of phagocytic research, but accelerated around the late nineteenth century and the turn of the twentieth century. Since then progress has accelerated still further. This chapter however aims to put our current progress into a historical context and to explore some of the interesting personalities who have set the ground work for our current understanding of the subject of this book, namely phagocytosis.

Keywords

Animalcules · The Phagocyte theory · Early observations of phagocytosis · Early experiments on phagocytosis · Early Microinjection experiments

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Introduction

In this chapter, the focus will be on the history of research into phagocytosis and some of the researchers who you may find interesting and important. As this a only "brief history", I will start with the advent of microscopy (since phagocytosis is a purely microscopic event) and will end around the inter-war period (ie 1920–1939) after which more "modern" research approaches were adopted. This end-date is of course an arbitrary decision. As is my focus on European and North America research. However, I apologise for any absence but unfortunately, I neither know of nor could comment on any Arabic, Asian or any other pre-1930 phagocytosis research. I am sure that it exists, but I have been unable to find any information on this (and would probably be unable to read it, if were pointed out to me). I therefore apologise to the descendants of those who have made significant contributions to understanding phagocytosis (from whatever continent of origin), but whom I have omitted.

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Purpose of Chapter

ln preparing this chapter, three things became immediately apparent.

1. The first is that collaboration and sharing of techniques and insights (sometimes unacknowledged) between seekers of true knowledge, is always a good thing. In the early stages of the history of phagocytosis there are many obvious examples where understanding leaps forward. For example, Leeuwenhoek (c 1674), rightly recognised as the Father of microbiology, discovered single cell organisms (animalcules) in "dirty" water using the simple of hand-held microscope described earlier by Robert Hooke in his book *Micrographia (*1665*).* Hooke himself preferred the compound (tube) microscope but when Leeuwenhoek wrote a letter to Royal Society in London describing aspects of the previously unseen microscopic world, Hooke was the one who replicated the observation (despite failure by another Royal Society Fellow, Nehemiah Grew, and by himself on two earlier occasions) and recommended its publication. What is especially remarkable was that the letters from Leeuwenhoek were written in Low Dutch which Hooke had to first to teach himself how to read (Lane 2015). All Leeuwenhoek's letters to the Royal Society were published in its journal, Philosophical Transactions (in English, having first been translated by the founding editor of the journal, Henry Oldenburg) within a few months of receipt (Lane 2015). Were Leeuwenhoek's observations not accepted and published, it is likely someone else may have done so later, but there would have been a slowing down of progress; and many others (unaware of Leeuwenhoek's findings) wasted their time by following older theories which were obviously absurd or untenable in the light of Leeuwenhoek's discovery.
2. The second is how the obvious acknowledgment of much progress in understanding cell biology has been made since the invention of the microscope: but the surprise is how little our current understanding has increased since the earliest papers. Many details of the molecular and biophysical process of phagocytosis, of course, are now understood and questions which could not have been previously asked have now been answered. These are, of course, the subject of subsequent chapters in this volume. But in the context of the big historical stories of phagocytosis research, it could be argued that these are but the necessary dotting of "i"s and the crossing of "t"s of a story already written. The earlier researchers had the fun of being constantly excited by the "remarkable" and undreamt of worlds and phenomena. It was of course much easier to discover that single cells can "eat" other smaller single cells (by simple observation) than to establish the molecular mechanism by which they do so. But it surprising to see the amazing ingenuity of the earlier researchers, who had no off-the-shelf technical kits and equipment solutions; and with these were able to discover intracellular details of the process such as the pH within the phagosome and the role of localised cytosolic Ca^{2+} changes (see later).
3. The third is that there really is no start-date for "history", especially in the history of scientific discovery. Every scientific advance depends on an earlier advance: which in turn depends on an even earlier one: and so on until we reach what is obvious or common knowledge. It is possible that somewhere in this chain, there was a "eureka" moment, on which later research was built. However, even then, I would argue that that "eureka" moment" arose to the "prepared mind" who already understood the state of knowledge to that point and so was suddenly able to see what had previously been obscured by confusion, in a sudden moment of clarity. But this clarity arose because of an observation or experiment. With Archimedes, it was a commonplace observation made by many before (ie everyone would know that a full bath would overspill as you get into it). With Metchnikov, it was an experiment that no one had done before (the thorn in the star-fish experiment – see later in this chapter) designed to test an hypothesis. However with both these

(and other) "eureka moments", the observation and the experiment were in the context of deep thought and an attempt to solve a specific puzzle. Many of the puzzles of phagocytosis have been solved, but many more remain.

The purpose of this chapter is therefore not only to set the historical background for the subsequent chapters, but also to re-tell some of the stories in the historical adventure which may inspire future imaginative researchers to see through the confusion of the thousands of papers published annually so that they may have their own "eureka" moment.

Let's Start at the Very Beginning

As Rodgers and Hammerstein wrote (1959), it is "a very good place to start". However, finding the "very beginning" of phagocytosis is not easy. It may be supposed that the "beginning" of research into phagocytosis would start with the naming of the process as being "phagocytosis". This term was first used by Ilya Metchnikov (1845–1916). Many reviews of the history of phagocytosis, not surprisingly, therefore start with Metchnikov. After all, he won the Nobel Prize in 1908 for his convincing work on his "phagocytic theory". He was, thus, undoubtedly of considerable importance to our story, as we will see later, and can be considered as the "father of phagocytosis". Olga Metchnikov, Ilya Metchnikov's second wife, gives an account of how the name phagocytosis arose in her biography of her husband, La vie d'Elie Metchnikoff (1920), later translated into English as The Life *of Elie Metchnikoff* (1921). She writes that:

> *On the way back to Russia through Vienna (in 1882), he (Metchnikov) went to see the Professor of Zoology, (Carl Friedrich Wilhelm) Claus .... and expounded his theory (ie the unnamed phagocytic theory) to them. They were much interested, and he (Metchnikov) asked them (Claus and his colleagues) for a Greek translation of the words "devouring cells" (or "eating cells"; or, in the German language which they were presumably speaking, "Fresszell"), and that is how they (the cells) were given the name of* ***phagocytes.*** *... It (as the "phagocyte theory") appeared soon afterwards in 1883* (ie Metchnikoff 1883).

The name which Claus came up with is derived from the Greek φαγεῖν (*phagein*) "to eat or devour", appearing as the first element "phago-"; and κύτος, (*kytos*) "a container"*, as the second element "cyte"* (now used as a designation for a number of cell-types eg leukocyte, lymphocyte, hepatocyte, adipocyte and many more). By extension of the "English" name for phagocyte (derived from Greek by a German for use by a Russian), the process these cells undertake is called **phagocytosis.**

But, despite having no name previously, Metchnikov was not the first to observe and describe the process of phagocytosis. In a masterly review, Thomas Stossel (1999) points to many earlier description of ingestion of particles by cells. Indeed, Metchnikov made his discovery that phagocytes in higher animals were immune cells, by first looking at the evolution of digestion, which was already known to occur in "lower animals" by phagocytosis. So it must be in "lower animals" that phagocytosis was first described. These "lower animals" are of course, single-celled animals (or animalcules, as they were called by Leeuwenhoek). There is a very accurate description of phagocytosis by amoeba published in 1875 (many years before Metchnikov's 1883 paper) by the American polymath Joseph Mellick Leidy (1823–1891; Fig. 2.9a). In 1853 (aged 30 years) he was elected Professor of Anatomy University of Pennsylvania, and later also President of the Academy of Natural Sciences of Philadelphia (where an impressive statue, 8 ft high on a 10 foot plinth still remembers him). As a palaeontologist and anatomist, he was an early supporter of Darwin's theory of evolution, ensuring Darwin's election to membership of the Academy. But Leidy was also a brilliant microscopist and in 1846 became the first person to use the microscope to solve a murder (Hare 1923) by showing that the blood on the murderers clothes was not the chicken blood that he had claimed. Under the microscope, Leidy could see that the red blood cells were not nucleated, as are avian red blood cells, and so

the murder's explanation could not be true. It was also as a microscopist that Leidy reported to the Philadelphian Academy his observation of how amoeba "swallow food" ie phagocytosis (Leidy 1875). Before Leidy's report, as we shall see later, it was already known that in the microscopic world, cells could swallow other smaller cells, but the mechanism was unclear. In Amoeba, it looked as if the "food particle" becomes stuck to the viscous gel which it was thought made up the amoeba's body and simply sunk into it, just as a hazelnut in contact with a viscous sugar syrup is at first stuck on the syrup surface and then sinks into it, to finally be encased in the syrup. However Leidy reported to the Academy that "he had repeatedly observed a large Amoeba . . . . . creep into the interstices of a mass of mud and appeared on the other side without a particle adherent". So the "sticky surface" theory did not seem to hold. He then reported what actually occurs during phagocytosis. His verbal report to the Academy, published in the third person, is as follows.

> ***On the mode in which Amoeba swallows its food*** *– Prof Leidy remarked that . . . . on one occasion he had accidentally notice an Amoeba, with an active flagellate infusorium, a Urocentrum, included within two of its finger-like pseudopods. It so happened that the ends of these were in contact with a confervous filament, and the glass above and below between which the Amoeba was examined, effectively preventing the Urocentrum from escaping. The condition of imprisonment of the latter was so peculiar that he was led to watch it. The ends of the two pseudopods of the Amoeba gradually approached, came into contact, and then actually became fused, a thing which he had never before observed in an Amoeba. The Urocentrum continued to move actively back and forth, endeavouring to escape. At the next moment a delicate film of the ectosarc proceeded from the body of the Amoeba, above and below, and gradually extended outwardly so as to convert the circle of the pseudopods into a complete sac, enclosing the Urocentrum.*

To anyone who has watched phagocytosis in real time, either by Amoeba or neutrophils, this all sounds very familiar and is a brilliantly accurate description of the event (and all from a single "accidental" observation!). The two "finger-like pseudopods" form the phagocytic cup holding the target, which gradually encroach around the particle. Then, the more rapid closure caused by the "*delicate film of the ectosarc",* which is also often seen. Leidy's report surely confirmed the view of that phagocytosis was not simply "swallowing" by sinking into a viscous inert cell body and showed that phagocytosis was, in fact, an active and complex process undertaken by the cell.

Twenty years earlier, Claparède (Fig. 2.9d) also reported the act by which amoeba phagocytose their prey (Claparède 1854). Edouard Claparède, a Swiss anatomist working in Geneva, where there also remains an impressive statue to him, reports his impression of phagocytosis as follows.

> *The amebas feed in a most remarkable way. They glide slowly along, attach themselves like snakes to the prey to be swallowed and, like a soft mist moving across a landscape, completely encircle it: one has the impression that the object still lies underneath, but it has already been enclosed within the body.* (translation by T.P. Stossel 1999)

Claparède use of the word "swallow" and the phrase "completely encircle it" suggests phagocytosis, but it is obviously a more romantic picture than that of Leidy. However, although it does not have modern day scientific rigour, and lacks the detail that might convince a sceptic that phagocytosis was an active process, (as does Leidy's description), it is easily recognisable as phagocytosis in action and another stepping stone in phagocytic research.

At this point in our journey into the past, it is important to point out that "the past" is truly a "foreign country" (Hartley 1953). When microscopes first revealed a world full of weird and wonderful "animalcules", there was a profusion and confusion of amazing animals. Almost anything could be believed. As well as amoeba, "dirty" water or water made "dirty" by leaving in contact with hay or soil (infusion) contained many other unicellular animals which were able to ingest food particles. One such group of animals are the heliozoans, also called sun-animalcules because they have stiff straight long projection (axopodia) made of micotubles which radiate from their spherical body (Fig. 2.1a) giving the appearance of a child-like depiction

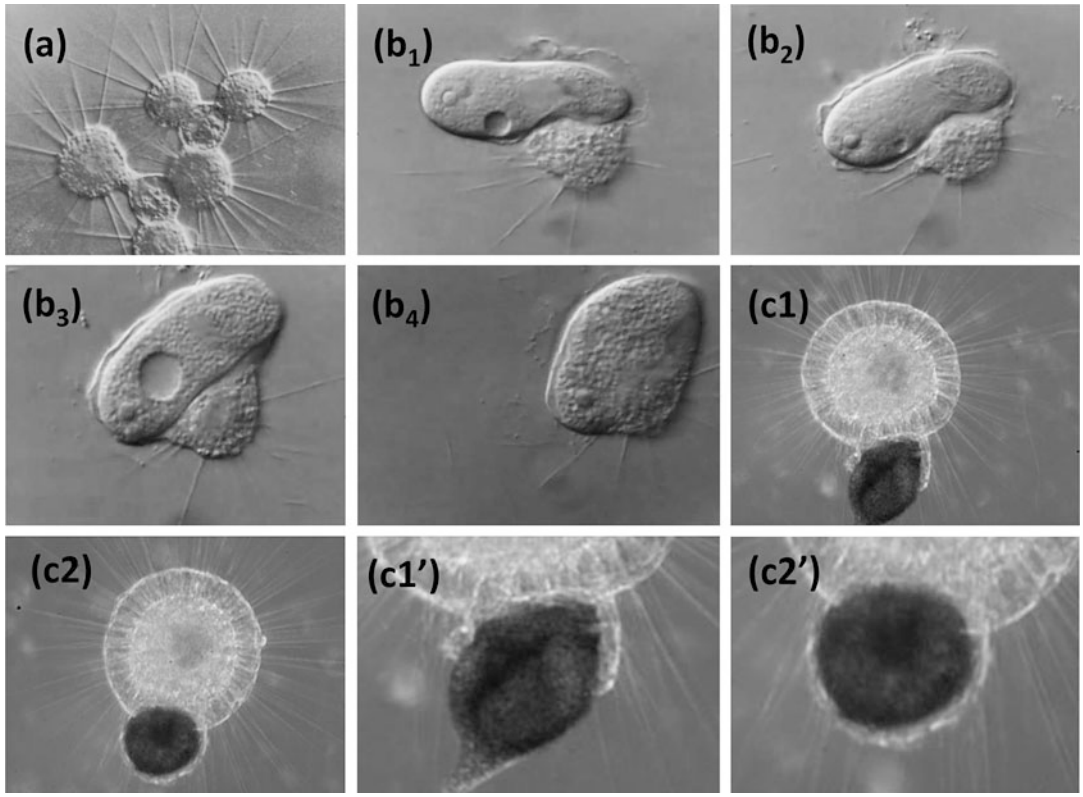

Fig. 2.1 Heliozoan Phagocytosis. (**a**) A phase contrast image showing a collection of heliozoans with typical spherical bodies and spines projecting to form "sun-like" shapes. Their size can vary from 20 μm to 1 mm in diameter. (**b1–4**) A sequence of images showing phagocytosis of a large paramecium by a heliozoan. (**c**) Images showing details of phagocytosis with (**c1**) and (**c1'**) showing the typical phagocytic cup and (**c2**) and (**c2'**) showing closure of the phagosome, with (**c1'**) and (**c2'**) at higher magnification. (The images are from the cell biological resource held by Institut für den Wissenschaftlichen Film Hausmann 1986)

of the sun. Surprisingly, they also undertake an amazing phagocytosis and can engulf targets of equal or greater size that themselves, once the prey has been immobilised by the spines (Fig. 2.1b). This type of phagocytosis can be slow, to complete (hence the need to catch the prey first on their spines), and can also involve a massive expansion of available cell surface area. This is achieved by exocytosis of granular membrane and, sometimes by recruiting other heliozoans, which together provide a joint phagosome which all partners can share. With smaller targets, phagocytosis procedes by the formation of a "classical" phagocytic cup (Fig. 2.1c). I have laboured this point, because it is the Heliozoan that provides the next major stepping stone. The Swiss scientist Rudolf Albert Kölliker (1817–1905), studying Heliozoans, made perhaps the first major description of phagocytosis. Kölliker (Fig. 2.9c) held some interesting posts, including in 1844 Professor Extraordinary of Physiology and Comparative Anatomy at Zurich University and in 1847 Professor of Physiology and Microscopical and Comparative Anatomy at University of Würzburg. Kölliker was later elevated to the nobility in 1897 by Prince Regent Luitpold of Bavaria for his scientific contributions and in later publications, he is consequently called Albert von Kölliker (the insertion of "von" signifying ennoblement). Why Kölliker is important to our story is that in 1849, 6 years before Claparède's romantic and 26 years before Leidy's careful descriptions of phagocytosis by amoeba, he had provided a detailed and accurate description of

phagocytosis by the heliozoan, Actinophrys. As this type of phagocytosis may not be as familiar as that of leucocytes or amoeba, Fig. 2.1 shows some examples, including from the movie available from the excellent archive of Institut für den Wissenschaftlichen Film (Hausmann 1986). With this in mind, we can see the accuracy of Kölliker's 1849 description (Kölliker 1849) given on page 202 of Zeitschrift für wissenschaftliche Zoologie, 1849 volume.

"The creature which is destined for food (ie trapped by the spines), gradually reaches the surface of the animal (ie the heliozoan, Actinophyrys), in particular, the thread that caught it is shortened to nothing, or, as it often happens, once trapped in the thread space, the thread unwinds from around the prey when close together and at the surface of the cell body". Here's what happens next:

> *The place on the cell surface where the caught animal is, gradually becomes a deeper and deeper pit (fig. 2f) into which the prey, which is attached everywhere to the cell surface, comes to rest. Now, by continuing to draw in the body wall, the pit gets deeper, and the prey which was previously on the edge of the Actinophrys, disappears completely, and at the same time the catching threads, which still lay with their points against each other, cancel each other out and extend again (fig 2g). Finally, the edges "choke" the pit, so that it is flask-shaped (flaschenformig) (fig 2g) all sides increasingly merging together, so that the pit completely closes and the prey is completely within the cortical cytoplasm (Rindensubstanz). Here it lingers for variable lengths of time (fig. 3f), but will always move towards the centre of the cell, and finally enters into the deeper part of the cell (fig. 3g), in order soon to find its finite fate within.* (translated into English with the grateful assistance of *Google translate* at translate.google.com)

It is clear that this was not a single or accidental observation because Kölliker goes on to follow the fate of the ingested prey. He writes that usually the ingested infusorium (a catch-all name for the animalcules which appear after water is left in contact with hay or similar) is completely dissolved and that "the space that sheltered it (ie the phagosome) is diminished and disappears completely." However, he also reports that

> On the other hand, if an indigestible remnant remains (a membrane of cellulose, a chitin skeleton, a shell of a lynceus, or a radiolarian (raderthierche) etc.), it simply re-emerges by contraction of the homogeneous cytoplasm (leibessubstanz) (fig. 3m), in the direction the object followed on entering, until it finally leaves the whole area, while the canal and the opening which led it out, disappear without a trace.

From the drawings that Kölliker made (Fig. 2.2), it can be seen that the size of the target is smaller than the example given in Fig. 2.1b and is thus, probably, more familiar to today's phagocytologists with a phagocytic cup or pit in the cell body clearly drawn. It is also interesting that in his Fig. 2.3, the internalised material is clearly within a spherical membrane within the cell, with a small clear (water) space around it. He was surely seeing the internal phagosome. Furthermore, unlike the ciliates, such as paramecium, which have an anatomically identifiable and permanent "oral groove" (vestibulum) ending in the cytstome (cell mouth) for ingesting food, the Amoeba's "mouth" is a transient pit " opening ....(and).... disappearing without a trace". Kölliker's published account of pseudopodia and the transient pit (phagocytic cup), and the visualisation of phagosomes is at least as accurate an account as given by Leidy 26 years later for phagocytosis by Amoeba. All the key elements of phagocytosis (as we understand it) are present in the Heliozoan. This may be, therefore, the first complete report of phagocytosis and is thus important for this alone.

But is this the "the very beginning"? Not quite.

The "swallowing" of food by single cells was known before Kölliker's report. For example, 10 years before Kölliker's paper, Andrew Pritchard (1804–1882) included some details which show that it was widely accepted that amoeba and other animalcules internalised food. Pritchard (Fig. 2.9b), an Englishman, was a member of a religious sect of Unitarianism which believed that "God and nature were one". He was also an optician/lens maker in London, UK, and had several shops in London that sold optical microscopes, microscopic accessories and microscopic preparations ready for use. He may thus have had a commercial, rather than purely academic, reason for publishing books such as

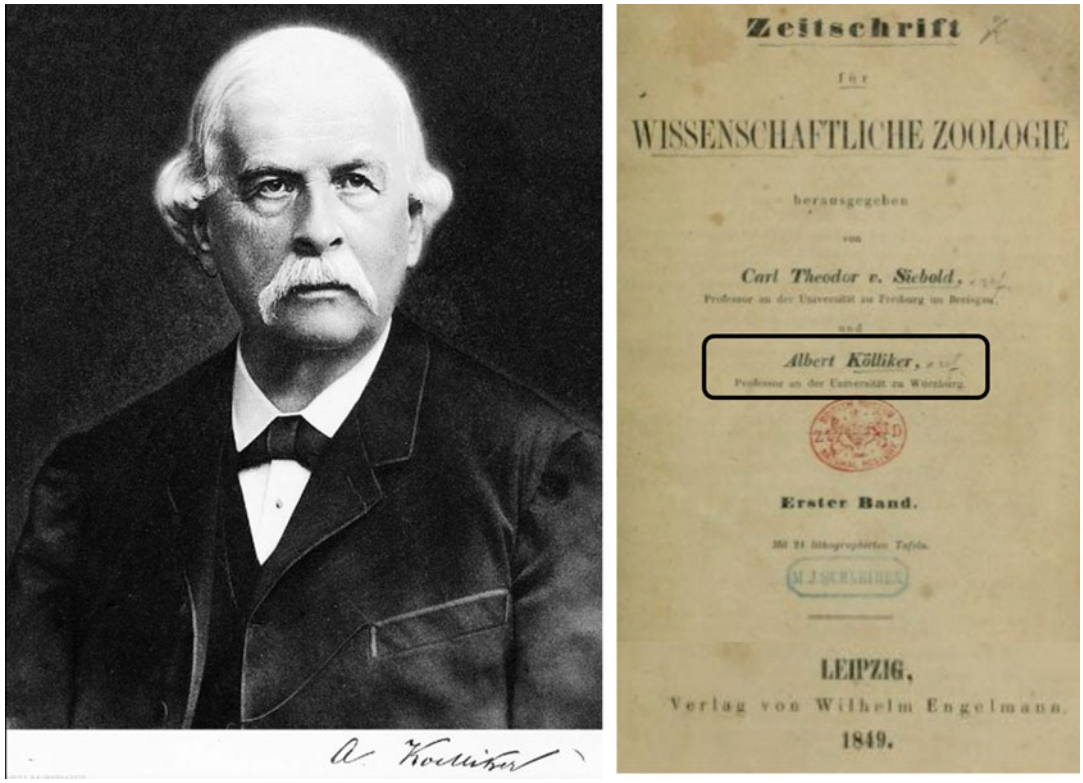

Fig. 2.2 Albert Kölliker. The image on the left shows a photograph of Albert von Kölliker and on the right the front cover of the ground–breaking report in 1849

"*List of 2000 Microscopic Objects*" (Pritchard 1835). But his book "The Natural history of animalcules" published the year before (Pritchard 1834) shows that he had a wide knowledge of the subject and is an important contribution to the history of phagocytosis. For example, he says of Vibrio punctatus (section 94): "*They are eaten by the Proteus diffluens and the large Vorticella which see*". In the same book, he earlier describes the process of "eating" by "Proteus diffluens", the melting Amoeba (Proteus section 22):

> *When in its contracted state, it (the amoeba) appears like a gelatine ball; this it readily changes, thrusting out branches of different dimensions in various directions. Some of its numerous forms are shown in the group, figures 8, 9, 10, 11 and 12. When it (the Amoeba) swallows animalcules which are covered with a crustaccous shell, as in figure 9, 10 etc, it accommodates its shape to the food. The mouth aperture is situated at the cross in figure 9.* (Pritchard 1834)

Pritchard tells us that "*the long animalcules within them (the Amoebae) are species of Bacillaria, which it has seized and eaten: they serve to exhibit the wonderful dilatation of their stomachs*". The part of the plate showing figures 8, 9, 10, 11 and 12 to which Pritchard refers in this paragraph are shown as Fig. 2.4a, b. Co-incidentally in Chap. 5 of this book, there are more modern examples of a similar phenomenon, together with an examination of the phospholipids signalling (as in Chaps. 3 and 9).

So in 1834, Pritchard, has nonchalantly stated (i) that Amoeba eat Vibrio, as if everyone already knew Amoeba ate other animalcules, and this was just a detail of its diet, (ii) the process of "eating" was by swallowing, and (iii) that the captured prey was clearly within the cytoplasm of the Amoeba. The detail that Amoebae "swallow" its food is suggestive of phagocytosis and since the objects swallowed are clearly inside the Amoeba,

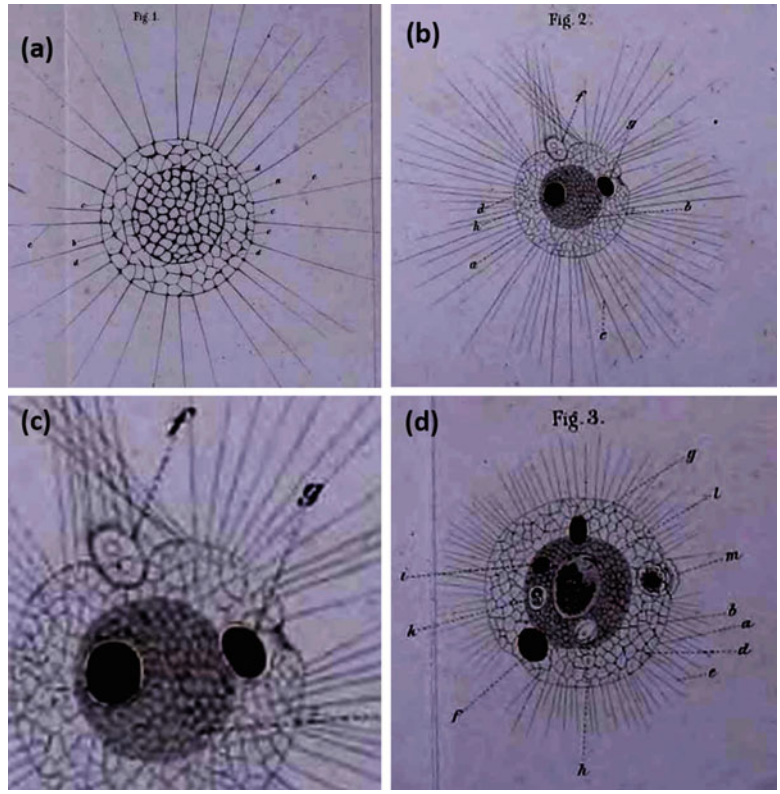

Fig. 2.3 Phagocytosis by Actinophyrs (Heliozian) as reported by Kölliker in 1834. The images show figures from the original paper with their original labelling. (**a**) Kölliker 's figure 1, (**b**) Kölliker 's figure 2 and (**d**) Kölliker 's figure 3 with their original labelling referred to in the paper by Kölliker (1849). (**c**) shows an enlarged view of Kölliker 's figure 2 to more clearly show two stages of phagocytosis (original label *f*) the "pit" or phagocytic cup formation and (original label *g*) internalised prey or phagosome. There were no scale bars in the original drawings

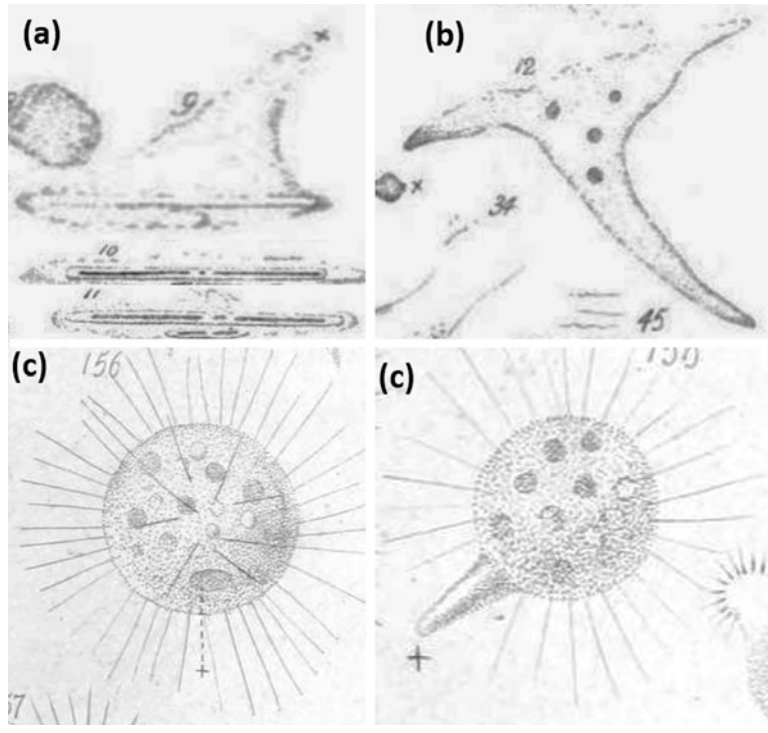

Fig. 2.4 Illustations from Andrew Pritchard's work (1834). (**a**) Shows "eating" by "Proteus diffluens" given in Proteus section 22 of The Natural history of animalcules" (1834), where the amoeba is distorted by the shape of the phagocytosed object "*covered with a crustaccous shell"*. (**b**) Shows the uptake of coloured particles ("*indigo, carmine or other minutely divided bodies")* within phagosomes within the amoeba. (**c**) Shows heliozoan phagocytosis with the entrance and exit points on the cell marked and (**d**) shows the "proboscis", probably an extended (and stylised drawing) of the phagocytic cup

changing shape to accommodate it, may thus represent one of the first reports of phagocytosis by Amoeba. It is interesting that Pritchard must have observed the entire process of phagocytosis (without comment) as he could mark the site of the "mouth aperture" (see Pritchard's fig 9 in our Fig. 2.4a, b). This terminology may be strange to our ears, but the "mouth aperture" must be where Pritchard saw the "lips" of the Proteus open and "swallow" the object. We know that this was actually phagocytosis and we would use terms like pseudopodia, but how could Pritchard describe it other than opening a "mouth aperture"? It is interesting that he does not say that Amoeba had a mouth (an anatomically distinction mouth like he ascribes to many of the ciliates for example), but simply that its food was swallowed via an aperture. The "stomach" that he mentions is surely the phagosome, but as he has no other word for it, he used the analogy of the human stomach. When he describes the amoeba equivalent of a digestive tract, it is obvious that he is not describing a stomach. He says that the Amoeba's "*digestive organs consist of a number of sacs*" and demonstrates this by using coloured dye particles that, when ingested, ended up in these vacuoles.

> *When they (amoeba) are fed on indigo, carmine or other minutely divided bodies, they (the digestive sacs) remain circular: several of these are shewn in figure 12.*

In Pritchard's figure 12 (our Fig. 2.4b), there are several discrete phagosomes, so presumably Pritchard was not implying a single stomach but that his coloured test objects were small enough not to distend the cell and that each ended up in its own vacuole. Pritchard also reports in section 235 that Trichoda Sol (Actinophyrs sol), the Heliozoan from which Kölliker described phagocytosis so clearly and completely, has *"as many as twenty polygastic sacs"*. He has trouble in accurately describing the way it feeds, but from his use of the word "suction", it is clearly something unusual.

> *This creature is an interesting object for the microscope: it preys upon other animalcules by suction and has been found attached to Kerona pulsulata. Size 1/900th of an inch* (1/900th of an inch = 28 μm)

Pritchard also had trouble in describing the apparatus for this suction and gives one credible and another incredible view of the "proboscis" (see Fig. 2.4c, d). This must be the extending pseudopodia, but the description is difficult to understand.

> *Its mouth is elongated into a proboscis, as shewn in fig 158; this the creature can contract at pleasure, and when turned towards or from the observer appears like an oval sac, as shewn by the dotted line and cross below figure 156*

Presumably, the "suction" which Pritchard attributes to the Heliozoan was actually the movement of prey towards the cell by the action of the spines: or even by being attached to extending pseudopodia which, without high magnification and phase contrast, may be invisible, so that when they contract drawing the prey towards the cell body, it looks like suction. Presumably, the proboscis in fig 158 is an exaggeration, being a simple geometrical shape. However, it may be based on what was observed. The prominent phagocytic cup of the Heliozoan was shown as early as 1784 (as we shall see later). The appearance of the mouth as an "oval sac" (in figure 156) is more realistic, and it is tempting to suggest that Pritchard was seeing the "pit" or phagocytic cup described by Kölliker.

Pritchard, who was obviously well-versed in the world of microscopic animalcules, showed by his use of coloured particles to "feed" Amoeba (and other animalicules), that he must have read (or be aware of) the earlier work of Gleichen. Gleichen first used dyes particles to convince himself, and others, that animalcules really did engulf their prey.

Baron Friederich Wilhelm von Gleichen-Russworm (1717–1783), to give him his full name, began his career in the army, reaching Lieutenant Colonel by 1748 (aged 31 years), and master of stables (senior equerry) 2 years later. In 1756 he resigned from the army and moved into the "family" castle of Greifenstein (Fig. 2.5a). It was here that, fortunately for science, he put aside the militaria and "focussed" on microscopy and experimentation. The results of his work were published in 1778 in his book entitled "Treatise on seeds and infusion, and on production; with

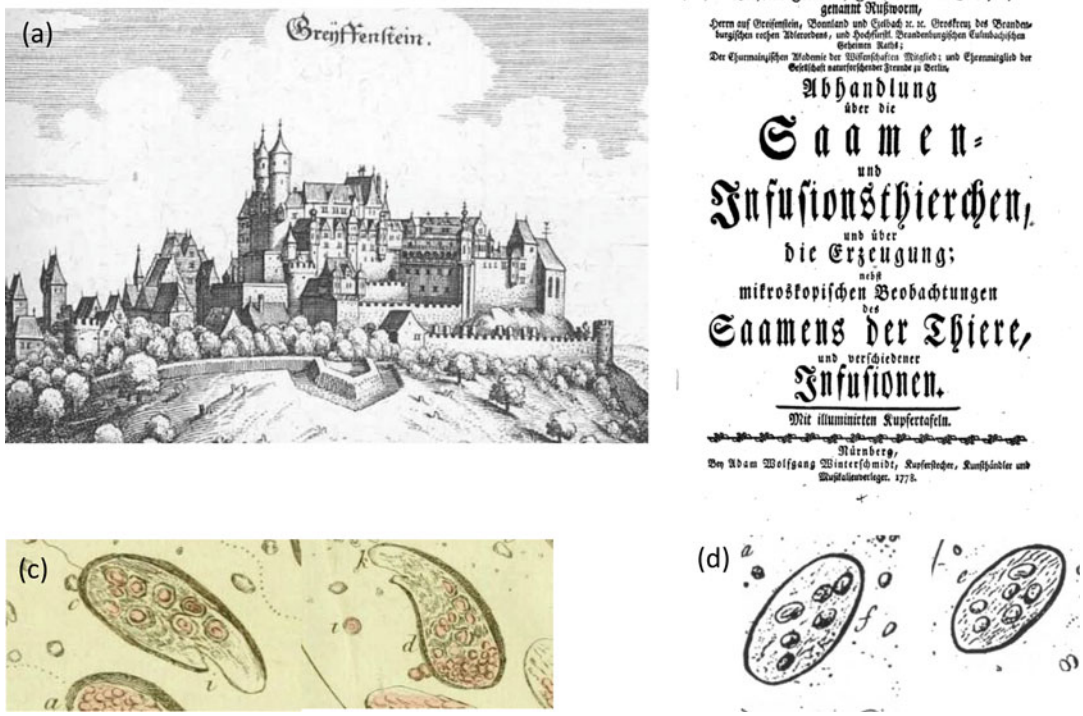

Fig. 2.5 The work of Baron Friederich Wilhelm von Gleichen-Russworm (1778). (**a**) The "family" castle of Greifenstein, where Gleichen underook his scientific studies. (**b**) the cover of his 1778 book "Treatise on seeds and infusion, and on production; with microscopic observations of the seed of animals, and various infusions" in which he reports important aspects of phagocytosis. (**c**) The uptake of red-coloured carmine particles was shown in Gleuchen's "pendeloques", which had "mouths". The clear space around the carmine is seen within the phagosomes. (**d**) Although not in colour, the drawing shows that there was no uptake of coloured particles by these animalcules which had no "mouths"

microscopic observations of the seed of animals, and various infusions" (Fig. 2.5b). In it, he explains why he is convinced that some of the objects he could see inside an animalcule which he calls "pendeloques" or pear-shaped pendants, which he thought might be unborn off-spring, were actually internalised food. He was convinced only after experimentation which he details in his explanation of Plate XXIIb entitled "Fressende Infusionsthierchen" and in the French translation "Voracious infusion animalcules" ie "Eating Infusion Animalcules". Having set up the objective of the experiment ie to test whether the vacuoles he could see in his "pendeloques" were internalised food, he continues:

> *So I coloured water with carmine, and I mixed it with an infusion of wheat, which contained many large "pear-shaped pendants" (pendeloques) and small "ovals", which lived there for some months. My expectation was fulfilled the next day; and I was not only convinced by the internal red colour of most animals, of an effective swallowing of food, but I also acquired more knowledge of their interior (4). This point, then, would be proved . . .*

Examples of the animalcules which Gleichen oberserved are shown in Fig. 2.5c, d. Now he knew the nature of the intracellular red particles, Greichen writes that

> *Henceforth I devoted all attention to the red pellets beneath my magnifier, ignoring all else*

Looking carefully at the intracellular red particles, he writes that

> *at first glance … you cannot avoid the idea that the internal pellets are eggs, because they are surrounded by clear rings as seen around frog eggs.*

This is seen in his careful drawings (Fig. 2.5c) and may have been caused by the osmotic swelling of the phagosome as the insoluble dye particle begins to breakdown in the acidic environment. Interestingly, Gleischen becomes convinced that the "pendeloques" were "schluckung" or, in the French translation, "deglutition" ie ***swallowing*** the food. The same description (swallowing) was used earlier by Leidy in 1875, Claparède in 1854 and Pritchard in 1834 in describing phagocytosis by amoeba, and it may be tempting to think this is what Greichen also meant. However, this term was probably used by Greichen because the "pendeloques" which took up the red particles had "mouths" (Fig. 2.5c) whereas other animals which failed to take up the particles did not (Fig. 2.5d). He writes

> *A careful inspection of animals c and d showed in both animals an incision at their narrow side (i and k) which resembles so much a mouth in shape and position that I truly think it is one.*

He also saw the indigestible dye particles being later ejected from "the *rear and once from the sides*", observations which he says "*required so much time, patience and visual concentration*" to convince himself that it really was happening. This may seem like a minor feature, but as a good scientist, he persisted with his "visual concentration" for 4 weeks and eventually saw the same event "10 to 12 times in innumerable observations" (ie n = 12 just to be sure!).

This is clearly excellent work. The drawing of the "pendolques", resemble Paramecium or similar cells which are "pear-shaped" and have a persistent oral groove. The mechanism by which Paramecium internalise particulate "food" is by drawing the particle into the mouth by beating cilia. Only once the food has been drawn down to the base of oral groove and in the vestibulum/-mouth cavity, does phagocytosis occurs. Thus Greichen's observations of internalisation of dye particles were the result of true phagocytosis; but unlike the Heliozoan, phagocytosis itself was out of sight at the base of the oral groove. This would make it difficult, without very good optics, to observe in real time. However a contemporary of Greichen, to whom he refers to several times in the book, Goeze, may have done so. The footnote to Greichen's report (possibly added after the main text was completed and containing Müller's classification name) states:

> (footnote 4) Goeze was fortunate enough to have before him, in a hay infusion, a quantity of Müller infusion animalcules, a counsellor of state, and described what he named as trichorda cimex, because of hairs (silk) whose body is lined in the anterior and posterior parts. According to what he says of their voracity and their ability to seize other infusion animalcules, it is a real carnivorous animal, in the microscopic world, that can be called wolf of *the infusion.*

Johann August Ephraim Goeze (1731–1793; Fig. 2.9e) studied theology at University of Halle in Germany, becoming pastor in several places in Germany before becoming the first deacon of the seminary of Quedlinburg in 1787, where he later died. Presumably, his hobby was zoology, and he undertook microscopical research in his "spare time" during which time he published, what is recognised as the first to describe tardigrades. Was he also the first to describe phagocytosis? Here is Goeze's description of what Greichen called the "ability to seize other infusion animalcules" (Goeze 1777).

> *On November 8, 1776 …*
>
> *Now I want to describe the predation scene in detail. As soon as the predator met one of the oval animals it suddenly dove at it and grasped it with the lips of its mouth which is located on its inferior side (number 8a). The captured animal defended itself as best it could. It struggled for a time in the jaws of the predator, especially if in an oblique position when seized. In that case the predator worked to turn the victim into a longitudinal position which was easier to accommodate to the tube which was its stomach. …. If the predator (number 7) seized the victim properly so that one end stuck in its throat, one might expect it to slide easily into the stomach. This is not what happens, however, in that the predator begins to choke and jerks itself backwards until the prey is fully swallowed.* (Translation from Stossel 1999)

It is not clear exactly what the predator (which Greichen thought was Tr*ichorda cimex*) was. Judging from the name given to it by Goeze,

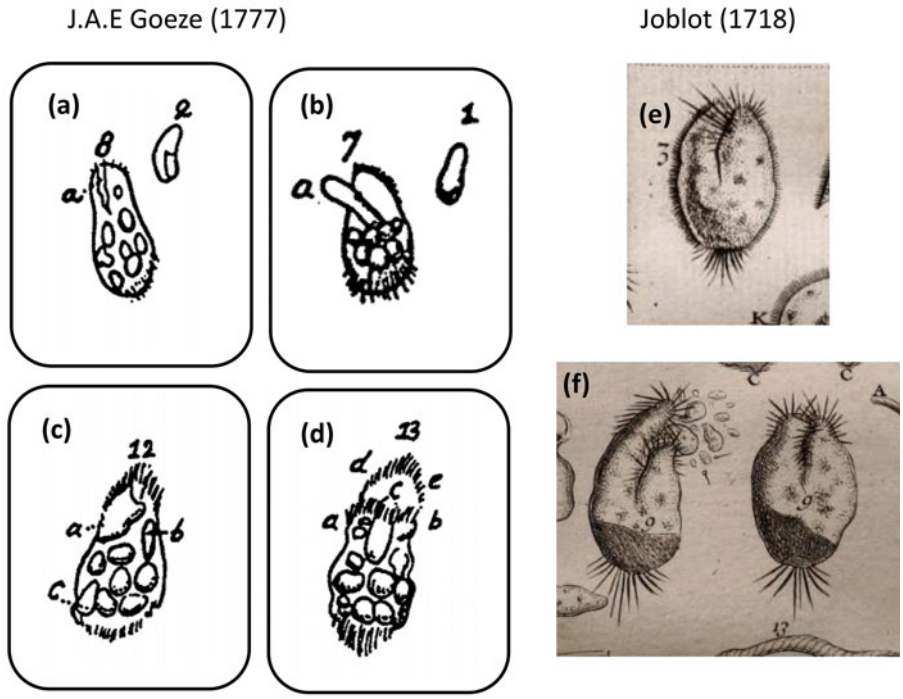

Fig. 2.6 Drawings of Devouring animalcules by Goez (1777) and Joblot (1718). (**a**–**d**) Drawings by Johann A.E. Goeze in his paper of 1777 showing Haarwanzen (hairy bugs) with "mouths" devouring prey. The animalcules are ciliated (hairy) and have permanent "mouths". (**e**, **f**) Drawings by Louis Joblot from his book "Observations d'histoire naturelle, faites avec le microscope" showing similar cilated and "mouthed" animalcules, which he called *la grosse Araignee aquatique ("the fat water spider")*

Haarwanzen (hairy bug), and the description in Greichen's footnote as having *hairs which "lined its body in the anterior and posterior parts"* together with Goeze's drawings (Fig. 2.6a–d), it is reasonable to assume it is a ciliate and thus similar to Greichen's "pendeloque" (Fig. 2.5d). The drawings referred as numbers 7 and 8 to in the quote above are reproduced here in Fig. 2.6a, b; (as are numbers 12 and 13 in Fig. 2.6c, d, which Goeze says "*showed an astoundingly voracious predator. As I watched it, it swallowed three oval animals*."). Like Greichen's description, the mouth is an obvious feature, and as with Greichen's report, this seems to be similar to oral groove of Paramecium. If this is the case, then the description by Goeze is of the seizure of the prey animal into the mouth rather than its phagocytosis into the cell cytoplasm. Goeze's statement that after the seizure of the prey, it is "fully swallowed" seems to reflect the prey being pulled down into the mouth (but still in the extracellular fluid) by the cilia hence the "hairy bug" appearing to "*choke and jerks itself backwards"*. Unfortunately, Goeze's description ends at this point and the phagocytotic event itself is not described. Perhaps the "the number 3 ocular and type A objective of (the) 'Composit' microscope" that he used did not have sufficient resolution or perhaps, Goeze thought it was "all over" after the "swallow". Greichen's dye particles, however, were internalised by the same or similar ciliates (see above and Fig. 2.7) showing that internalisation by phagocytosis had occurred. Goeze elegantly showed that the prey he was observing ended up within the "hairy bug" (and were still alive) by squashing the "hairy bug" to breaking point and allowing the internalised prey to escape. He reports:

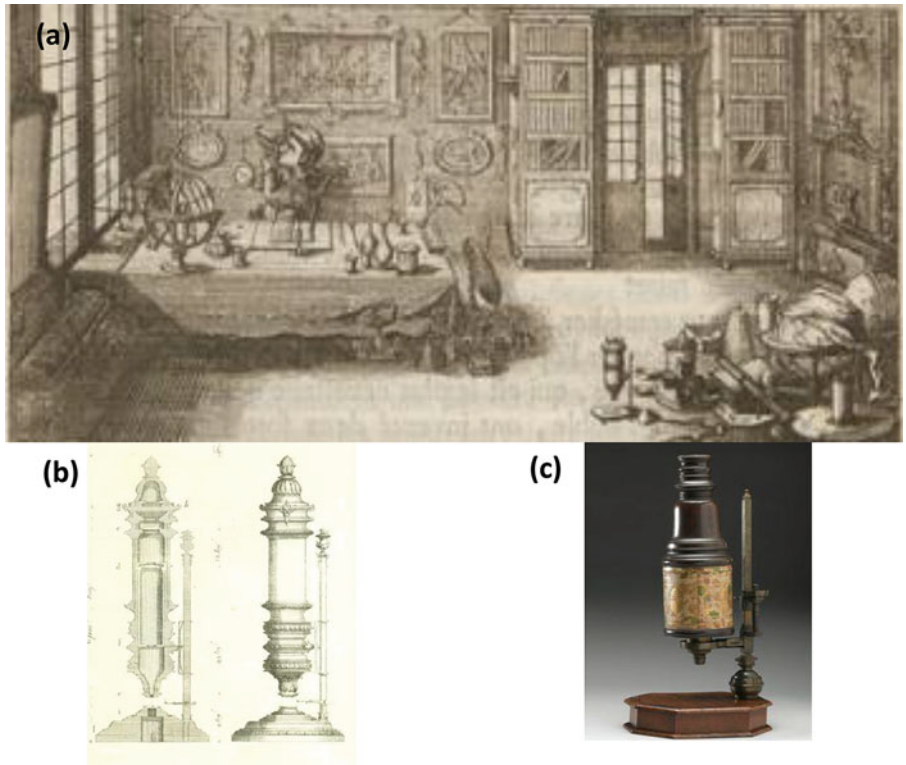

Fig. 2.7 The laboratory of Louis Joblot (1718). (**a**) shows a view of Joblot's laboratotry taken from the frontis peiec of his 1718 book "Observations d'histoire naturelle, faites avec le microscope". It shows books, microscopes and other items difficult to identify housed in a classical eighteenth century room within the Royal Academy of Painting and Sculpture in Paris. (**b**) Optical diagrams from Joblot's book showing improvements made by Joblot to the standard microscopes of his day. (**c**) A microscope of similar age and design to that shown in the frontispiece image if Joblot's lab

I must mention another experiment providing evidence that the hair bugs actually swallowed the oval animals. I applied a droplet to the lower plate of the recently devised Hoffman press with screws and small watch-springs–I will describe the device at another occasion—and superimposed the other plate, screwing it down until it touched the surface the drop. Then I gave the predators time to function and devour. One swallowed five small animals one by one, and they all were visible in its belly. Then I screwed the plate down further which caused the predator to become quiescent, whereas the swallowed animals still moved about within its body. By gentle further application of the screw, I gave them a final squeeze that caused the predator to burst, releasing the swallowed animals to freedom. The liberated animals immediately continued swimming in the liquid. I was delighted to be the deliverer of those swallowed victims, even in the microscopic world.

The last sentence seems to reflect Pastor Goeze's religious upbringing, although the zoological morality of killing one animal to save another is obviously complex. The experiment is, however, a very early example of the power of microscopic manipulation of cells to provide compelling evidence. Goeze in 1777 said of his micro-press that "*This is convincing proof of the benefits of this excellent device for microscopical experiments which otherwise would be impossible."* His foresight was good and future "excellent devices" such as micro-manipulation, optical tweezers, microinjection and advanced microscopic imaging would indeed provide "convincing proof" of cellular events during phagocytosis "*which otherwise would be impossible."*

For completeness, we should notice that Goeze referenced Joblot when describing the seizure of oval cells by his "peneloques". Louis Joblot (1645–1723) was French polymath and the first French microscopist. He was probable inspired by Huygens' visit to Paris in 1678 when Huygens demonstrated infusoria before the Academy of Sciences. Despite his obvious scientific interest, Joblot, from a well-off merchant family, in1680 accepted an unpaid appointment as assistant professor of mathematics at the Royal Academy of Painting and Sculpture in Paris, before becoming a full professor in 1699 (and receiving a salary). Joblot published his important book, "Observations d'histoire naturelle, faites avec le microscope" (Observations of natural history made with the microscope, 1718) while at the Academy of Painting and Sculpture (see Fig. 2.7a). In it, he described in detail some improvements to the then existing microscopes, including diaphragms in compound microscopes to correct for chromatic aberration (Fig. 2.7b). He also described and drew beautifully, but imaginatively, the animalcules he saw and named (also imaginatively).

Goeze (1777) wrote, as follows:

> *The Privy Councillor did not cite an authority, but I believe it correct to see these animals in the figures of Joblot, volume 1, P. 11, tables 2.f.3 and 8.f.9.9, which he calls la grosse Araignee aquatique ("the fat water spider") on p. 78. He also mentioned that they devoured Cornemeases ("bagpipes"). The figures, however, are a bit unnatural, as is typical for Joblot's pictures.*

The "fat water spider" is probably the same as Goeze's "hairy bugs" and Müller's Trichod cimex; although Müller identifies it with Joblot's "Pettit *Araignee aquatique" ie* small water spider). Joblot describes the encounter between his "fat water spider" and its prey in his book Observations d'histoire naturelle, faites avec le microscope (Observations of natural history made with the microscope) dated December 1714 (part 2 page 78)

> *(The fat water spider) approaches the figure of an oval cell; and its slightly squeezed mouth sometimes seems split up to the middle of its body, its lips are filled with small moving hairs, whose speed seems to be communicated internally to a small body which is perhaps the heart etc … (The fat water spider) feeds on other smaller fish, which we have called Bagpipes, and which seem to move in their bodies for some time.*

The drawing are, as Goeze correctly said, "a bit unnatural" but clearly show the similarity to Goeze's "hairy bugs": its mouth seemingly to split up the body, the lips with moving hairs (Fig. 2.6e, f). However, as with Goeze's description, it seems that Joblot is again seeing only the seizure of the prey and not phagocytosis itself.

We must now bring in Otto Frederik Müller, (1730–1784) a contemporary of Goeze. Goezez wrote that on seeing the carnage brought about by the "hairy bugs" on the "oval bugs" that "*At first I could not believe my eyes, because my mind recalled the works of Mr.* Müller *in "Histor. Verm Vol. 1. p.2.p.88" declaring "Nee ullus oculatior animalcula revera ab animalculis devorari vidit""* (ie no observer really saw an animalcule devour others). It is unclear why Müller made this statement but the use of the Latin word "revera" (in reality) suggests that he was questioning what had been was observed was animalcules actually eating other animalcules. This suggests that he thought the observations of others earlier than Müller and Goeze (eg Joblot) were of prey capture rather than "devouring". What Müller will be remembered for is his book "*Vermium Terrestrium et Fluviatilium, seu Animalium Infusoriorum, Helminthecorum, et Testaceorum non Marinorum, succincta Historia* (1773), in which, for the first time, he arranged the "infusion animalcules" into a logical genera and species. Müller (Fig. 2.9f), a Dane, initially trained for the Church, but was never ordained. Instead, he travelled European for a few years before settling down in Copenhagen with a wealthy wife. Presumably, having a such a wife relieved him of the need to earn a living, and he took up zoology and microscopy as "hobbies" but it is obvious that they became an obsessions. His new classification made descriptive but ambiguous terms (like "pendeloques" and "hairy bugs") obsolete and enabled future progress by ensuring that similar animalcules studied by different workers were or were not referring to the same animalcule. Müller tries to back-date the new classification by giving

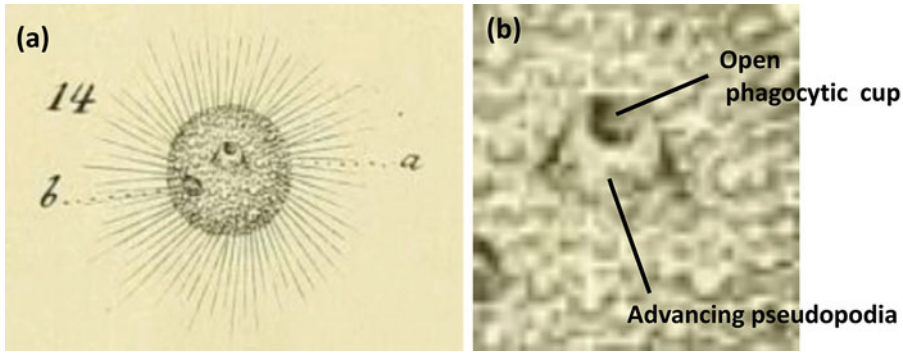

Fig. 2.8 O.F. Müller's phagocytic cup (c 1777). (**a**) Shows Müller's drawing of a heliozoan in the process of consuming the animalcule, Lynceus. The labels on the original drawing are shown and are labelled as showing "at the centre a raised nipple open, transparent (pellucentremque) that consumed Lynceus." where original label "*a*" is "papilla oris" or nipple mouth and "*b*" is "Insectum devoratum" or insect drained or devoured (presumably the shell remains of the Lynceus. (**b**) shows a closer view of the drawing of the "nipple mouth" which is probably a phagocytic cup with advancingpseudopodia, as labelled

the older names when he could. For example, the animalcule which Goeze called the "hairy bug", Greichen called by the new classification name "Trichorda cimex", which Müller tells us was called "petite araignee aquatique"("little water spider") by Joblot. So, in theory, data from different scientists could be cross referenced; eg Goeze's "hairy bug" was Joblot's "little water spider". The problem was that many animalcules look similar and, without photography, reliance must be placed on the skill of the drawing and the verbal description. In the book published in 1786, after Müller's death, "Animalcula infusoria fluviatilia et marina" useful illustrations are included and some animalcules, such as in section 177: Trichoda Sol, the Sun animalcule (Heliozoa Sol or Actinophrys Sol), which earlier featured in our story, is easily identified from the drawings in Müller book (Fig. 2.8b). Müller gives some stories and anecdotes (in difficult Latin) under each heading. He included this under Trichorda Sol the following:

> *Brunswigg ae aestate anni 1777. amicissimus wagner, me praesente, Lynceum ex interaneis expressit, hinc animalculum, licit maxime deses cohabitantia devorat*

Google Translate says this means something like "Brunswigg the summer of the year 1777. Wagner my great friend, (said) in my presence, Lynceum from intestinal worms, on the side, that little creature, although the most lazy, dwell with them then is eaten by them", ie looking at the Heliozoan (as a lazy creature) and the Lynceum (on the side) that live with them, Wagner remarks something like, "they (lynceus) live with them (Heliozoan) until they eat them!". This makes more sense when one looks at the accompanying drawing of a Heliozoan with a "*drained/devoured*" body of a Lynceum indicated (Fig. 2.8a). Lynceum is a radiolarian with an indigestible "shell" as had been seen by Kölliker. There seems to be no doubt that this is a depiction of Heliozona "eating"

The description of the accompanying drawing is

> *Fig 13 Trich. Solem, centro clauso,*
> *14 eundem centro papillam elevato ac aperto, pellucentremque devoratum Lynceum*

This translates as:

> Fig 13 Trich. Sun, the centre is closed,
> 14 The same, with at the centre a raised nipple open, transparent (pellucentremque) that consumed Lynceus

Müller's figure 14 is shown in our Fig. 2.8a with the labelled a, and b are given as a "(a) papilla oris and (b) Insectum devoratum" translated as "(a) nipple mouth and (b) insect drained or devoured". The drained or devoured remains of the "insect" ie Lynceus is shown inside the Heliozoan. It is

an amazing foreshadowing of Kolliker's report, where his Heliozoan also phagocytosed Lynceum leaving behind "the shell of a lynceus". What is more remarkable is that in fig 14, the "nipple mouth" (papilla oris) is shown in 3-dimensions (as Pritchard had attempted in 1834, shown as Fig. 2.8b). Müller shows this as not just a "pit" as described and shown in Kölliker's drawings, but has a projecting pseudopodia forming a complete phagocytic cup (Fig. 2.8b). Müller does not give a description of phagocytosis, but the drawing and its labelling is an important point on the graph tracking the history of phagocytosis as it may be the first observation of extending pseudopodia forming the phagocytic cup.

And so the journey ends, as Tolkein probably wrote. The path was winding, with many unexpected turns. But this usually is the way with scientific advance, even today. To summarise this attempt to "drill down" into the mass of old, multilingual writings, there was no single "Eureka" moment, but a gradual progress with observations being repeated and assumption bring reinforced until gradually it becomes the "obvious". Some of the people involved are shown in Fig. 2.9. Perhaps Leidy, Kölliker and (surprisingly) Müller should step forward a little and take a bow in the Awards ceremony; Leidy for the best early description of phagocytosis: Kölliker for the first description of true phagocytosis: and Müller for the first depiction of the phagocytic cup. All these important aspects of phagocytosis, were chanced upon, and the implications not really explored. But they were reported accurately and so remain as markers in our phagocytic history. Of course, this was still not "the very beginning",

Fig. 2.9 Faces of some early phagocytologists. (**a**) Joseph Leidy (1823–1891: aged about 40 years) (**b**) Andrew Pritchard (1804–1882: aged about c 46 years) (**c**) Albert Von Kölliker (1817–1905 aged about 42 years) (**d**) Édouard Claparède (1832–1871; aged 28 years) (**e**) J.A.E. Goez (1731–1793; aged 55 years); (**f**) Otto F. Müller (1730–1784: aged about 30 years)

as these pioneers would not have had that chance to observe anything had Hooke not publicised the new Microscope in 1665, and Leeunkook had not excited the world with his animalcules (1674). Perhaps the invention of the microscope marks the "very beginning"'; but that is another story.

The Eureka Moment of Metchnikoff

We must next turn to Metchnikoff (1845–1916). He took phagocytosis from being an interesting oddity of animalcules only of interests to (rich) amateurs, to an important event of the immune system in multicellular organism and especially in mammals. So much has been written about Metchnikoff (Fig. 2.10a), that I feel it is unnecessary to give only but the barest outline and so direct readers to recent (and older) reviews of Metchnikoff's work including those by Aterman (1998), Tauber (1992; 2003), McGonagle and Georgouli (2008), Cavaillon and Legout (2016) and Korzha and Bregestovskic (2016) and Gordon (2008, 2016). It should be noted that there are many English and French variants of his Russian name Илья Ме́чников, including "Metchnikov/Mechnikov/Metchnikoff/Metschnikoff/Mecznikow.", with his first name "Ilya/Elie/Ellie" with a corresponding initial E. or I. (This can add confusion and lead to omissions when searching for the work of this important phagocytologist, especially if relying only on electronic searching.).

Metchnikov himself is an interesting and well-travelled European. The biography of Metchnikov given to accompany his Nobel Prize Lecture in 1908 (Nobel Media Biographical 1908) states the he was born "in a village near Kharkoff", Russia, to "an officer of the Imperial Guard", who also owned land in the Ukraine steppes. We are told "even when he was a little

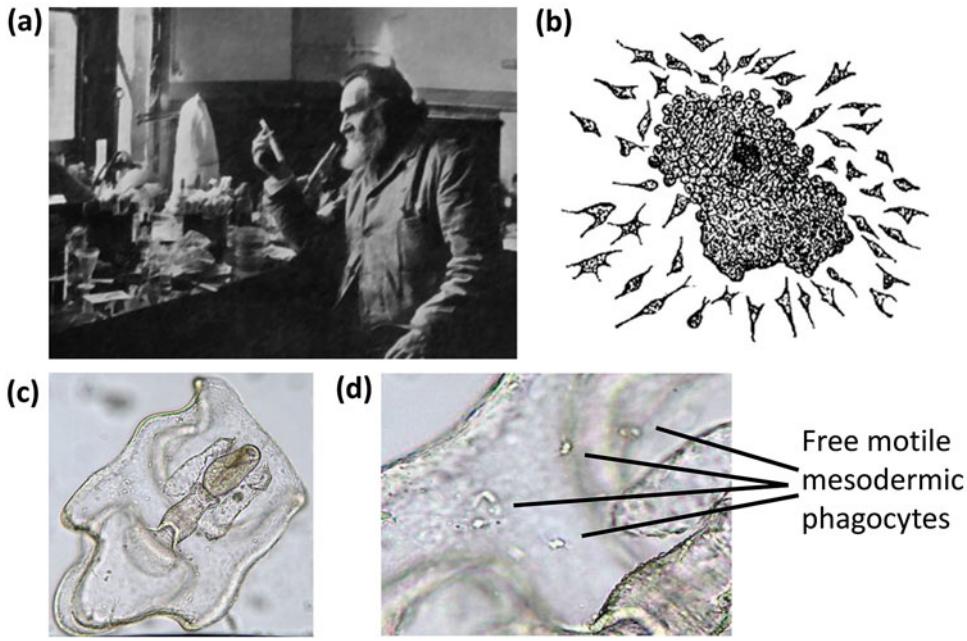

Fig. 2.10 Metchnikov. (**a**) The photograph shows Metchnikov in his laboratory at the Pasteur Institute, Paris, with his microscope behind him on the lab. bench. (**b**) The drawing (Fig. 32) in "Lectures on the Comparative Pathology of Inflammation" (Metchnikoff 1893) showing the result of the "thorn in the starfish" experiment. After injury of the starfish embryo, motile mesodermal pahgocytes were seen to accumulate at the site of injury. (**c**) The "starfish embryo", Bipinnaria asterigera, which has few internal organs and is as "clear of water". (**d**) The starfish embryo at higher magnification showing the free moving mesodermal cells clearly visible within the tissue of the embryo

boy, (he was) passionately interested in natural history, on which he used to give lectures to his small brothers and to other children". He went to the University of Kharkoff to study natural sciences, and completed the 4 year course in just 2 years. He then went to several European universities to undertake zoological studies and research. His doctoral thesis, submitted to Naples University, was on the embryonic development of the cuttle-fish and he then returned to Russia to take up a post as docent at the new University of Odessa and was duly promoted over subsequent years to the post of "Titular Professor of Zoology and Comparative Anatomy".

So far, Metchnkov's biography is a straightforward CV of a hard-working and driven young scientist. However, in typical Russian tragic style, his life story now takes several dark turns before he finally reaches his Eureka moment.

Olga, Metchnikov's second wife tells us in her book, "The life of Elie Mechnikoff" (Metchnikoff 1921) that in St Petersburg, Metchnikov "*was devotedly fond of B's children, whom he used to take for walks on Sundays and to the theatre now and then; he was always ready to read to them and to indulge them in every possible way*." In fact, Metchinkov's devotion to the children extended further than Sunday walks. Olga goes on to tell us that Metchnikov "*continued to entertain the dream of marrying one of them (the children) someday, and was particularly interested in the eldest, a girl of thirteen, intelligent, gifted, and lively*". Metchnikov's mother was not overjoyed by her son's choice when Metchnikov told his mother about the young girl, Ludmilla, judging by the letters which Olga published in her book. Things get even worse when it is obvious that Ludmilla is seriously unwell. Olga writes:

> *As Elie learnt to know his fiancée better, he became more and more attached to her. Their happiness seemed likely to be complete, but a cruel Fate had decided otherwise. The girl's health was not improving: her supposed bronchitis was assuming a chronic character (*it was probably tuberculosis *MBH). Yet the marriage was not postponed, and the bride had to be carried to the church in a chair for the ceremony, being too breathless and too weak to walk so far*. The marriage ceremony of the bearded Metchnikov to his a young invalid bride thus took place. It was clear however that "cruel Fate" had not finished yet.

Olga writes that after the wedding

> *Elie did his utmost to procure comforts for his wife, and hoped that she could still be saved by care and a rational treatment. It was the beginning of an hourly struggle against disease and poverty; his means being insufficient, he tried to eke them out by writing translations. His eyesight weakened again from overwork, and it was with atropin in his eyes that he sat up night after night, translating. There was but one well-lighted room in his flat, and he turned it into a small laboratory for the use of his pupils; his own researches he had to give up, his time being entirely taken up by teaching and translations.*

Ludmilla's health fluctuated and her inevitable death hit Mechnikov hard, as Olga describes:

> *When Metchnikoff went back to his wife he found her with eyes wide open and so full of mortal anguish and utter despair that he could bear it no longer and went out hastily, not to show her his dismay. This was his last impression; he never saw her again … Only half conscious, he walked up and down the drawing-room, opening and closing books without seeing them, his mind full of disconnected pictures ….. Time passed without his realising it. Then his sister-in-law came to tell him that all was over. This was on the 20th April 1873*

Metchnikov did not attend his wife's funeral and sank into a dark depression.

> *After the catastrophe, Metchnikoff felt incapable of thinking of the future, his life seemed cut off at one blow; he destroyed his papers and reserved a phial of morphia, without any settled intention. …. He said to himself: "Why live? My private life is ended; my eyes are going; when I am blind I can no longer work, then why live?" Seeing no issue to his situation, he absorbed the morphia. He did not know that too strong a dose, by provoking vomiting, eliminates the poison.*

And thus Metchnikov survived his suicide attempt. Olga tells us, thankfully, that eventually "*his thoughts turned towards Science; he was saved; the link with life was re-established.*"

In fact, although Metchnikov "was saved", there was still a little more tragedy yet to come. In Odessa, he lived in a flat below Olga's family; "*we were eight children, our ages ranging from one to sixteen years*" she writes. Metchnikov

"*having heard that I (Olga) was interested in natural science, it occurred to him to offer to give me lessons in zoology. I was delighted. He asked and obtained permission from my parents, and we eagerly set to work.*

This was part of a plan by Metchnikov to have the"ideal scientific wife" because Olga writes that:

> *Elie, being strongly attracted by me, returned to his former idea of training a girl according to his own ideas and afterwards making her his wife.*

However, Metchnikov was forced to marry Olga before he had "fully trained her". Olga writes that Metchnikov "*might have realised his programme of completing my education first and marrying me afterwards if he had not been prevented by the complete lack of accord between his ideas and those of my father. .... Elie decided to ask for my hand without further delay*". Olga leaves to the imagination of the reader what the "*complete lack of accord between his (Metchnikov's) ideas and those of my father"* was about, but Metchnokiv gets his way and the marriage is arranged. Olga's poignant description of the day of her wedding, highlights the lack of her maturity and the age difference between them.

"*Our marriage took place in February 1875; it was a very cold winter and the ground was covered with a thick coating of glistening snow. A few hours before the ceremony my brothers came with a little hand sledge to fetch me for a last ride. "Come quick," they said, "this evening you will be a grown-up lady, and you can't play with us anymore!" I agreed, and we rushed out to the snowy carpet which covered the great yard of our house. In the midst of our mad race my mother appeared at the window; she had been looking for me everywhere and was much disturbed. "My dear child! what are you thinking of? It is late, you have hardly time to dress and to do your hair!" "One more turn, mother! It is the last time, think of it!" Other childish emotions awaited me; my wedding-dress was the first long dress I had ever worn, and I feared to stumble as I walked. Then, too, I was frightened at the idea of entering the church under the eyes of all the guests. My little brother tried to reassure me by offering to hold my hand, and my mother made me drink some chocolate to give me courage.*

Elie was awaiting us at the entrance; my shyness increased when I heard people whispering around us, "Why, she is a mere child!" The ceremony took place in the evening, after which Elie wrapped me carefully in a long warm cloak and we set off, the sledge gliding like the wind, towards our new home. In spite of the day's emotions, I rose very early the next morning in order to work at my zoology exercises and to give my husband a pleasant surprise. He was now free to superintend my education, a very difficult and delicate task when having to do with a mind as unprepared for life as mine was.

This marriage seemed to bring stability to Metchnikov's scientific life, but there were suggestions in Olga's writing that, in the early days, Olga may not have been completely happy (she met some younger perhaps) because she writes "*At a certain time, Elie, believing that happiness called me elsewhere, offered me my liberty, urging that I had a moral right to it. The nobility of his attitude was the best safeguard*". However, she continues that *"As years went on, our lives became more and more united; we lived in deep communion of souls, for we had reached that stage of mutual comprehension when darkness flees and all is light.*"

But Russia was a dangerous place to be in the 1870s and 1880 and conspiracies and reprisals were a constant worry, which combined with a clamp-down on travel trips by the University, caused Mechnikov extreme anxiety which led him, once again, to consider suicide. This time, writes Olga,

> *In order to spare his family the sorrow of an obvious suicide, he inoculated himself with relapsing fever, choosing this disease in order to ascertain at the same time whether it could be inoculated through the blood. The answer was in the affirmative.*

Fortunately, Metchnikov's suicide failed once again and he fully recovered. Perhaps "cruel fate" now relented, and from then on, things took a more positive turn. As is often the case, wealth provided the answer. Metchnikov inherited finance from Olga's parents and was thus freed

from the University rules and especially its travel ban. Olga writes:

> *Thanks to my parents' inheritance, he was … to live henceforth independently. He wished to pursue researches on the shores of the Mediterranean: therefore, in the autumn of the year 1882, we went to Messina with my two sisters and my three young brothers. The children were no trouble to Elie, who loved them; on the contrary, he enjoyed organising the journey and arranging all sorts of pleasures for them.*

The importance of this release from University drudgery and its restrictions, allowing Mechnikov to go to Messina cannot be overstated. Metchnikov himself said

> *… it was in Messina that the great event of my scientific life took place. A zoologist until then, I suddenly became a pathologist. I entered into a new road in which my later activity was to be exerted.*

In order to understand this great event, we have to briefly follow Mechnikov's scientific thought processes. In his pre-doctoral days, in 1865 at the University of Giessen, he discovered intracellular digestion in one of the flatworms. As we have seen, it was well established that in single cell animals, digestion occurs after phagocytosis in "digestive vacuoles" within the cell. In higher animals, there are specialised structures, eg the gut, in which digestion occurs. In between the single cells and mammals, however, there are "intermediate examples". For example, in coelenterates there is a "gastic cavity" lined with cells that can also phagocytose. As this was the era of Darwin and the new ideas of evolution were in the air, Metchnikov's long-term project was to discover how it was possible that the gut in higher animals evolved from the unicellular animals with no gut and whether the evolution from intracellular digestion to intestinal digestion had other consequences. Olga tells us what was going on in Metchnikov's mind at that time:

> *The study of medusæ and of their mesodermic digestion confirmed him more and more in the conviction that the mesoderm was a vestige of elements with a primitive digestive function. In lower beings, such as sponges, this function takes place without being differentiated, whilst with other Cœlentera and with some Echinoderma the endoderm gives birth to a digestive cavity; yet, the mobile cells of the mesoderm preserve their faculty of intracellular digestion. As he studied these phenomena more closely, he ascertained that mesodermic cells accumulated around grains of carmine introduced into the organism.*

The last sentence about "grains of carmine" shows that Metchnokov knew of Greissen earlier work using coloured particles, and carmine in particular, being taken up into the phagosome of phagocytic animalcules (see above). Clearly Metchnikov's mind was prepared and he now had the freedom to follow a crazy idea. The time was ripe for his "Eureka moment": and here it is (in his own words):–

> *I was resting from the shock of the events which provoked my resignation from the University and indulging enthusiastically in researches in the splendid setting of the Straits of Messina.*
>
> *One day when the whole family had gone to a circus to see some extraordinary performing apes, I remained alone with my microscope, observing the life in the mobile cells of a transparent starfish larva, when a new thought suddenly flashed across my brain. It struck me that similar cells might serve in the defence of the organism against intruders. Feeling that there was in this something of surpassing interest, I felt so excited that I began striding up and down the room and even went to the seashore in order to collect my thoughts.*
>
> *I said to myself that, if my supposition was true, a splinter introduced into the body of a starfish larva, devoid of blood-vessels or of a nervous system, should soon be surrounded by mobile cells as is to be observed in a man who runs a splinter into his finger. This was no sooner said than done.*
>
> *There was a small garden to our dwelling, in which we had a few days previously organised a "Christmas tree" for the children on a little tangerine tree; I fetched from it a few rose thorns and introduced them at once under the skin of some beautiful star-fish larvæ as transparent as water.*
>
> *I was too excited to sleep that night in the expectation of the result of my experiment, and very early the next morning I ascertained that it had fully succeeded.*
>
> *That experiment formed the basis of the phagocyte theory, to the development of which I devoted the next twenty-five years of my life.*

This was a true "Eureka moment". The phagocytic cells within the starfish larvae moved to the site of injury as shown in his Eureka diagram (Fig. 2.10b). He repeated the experiment with different

stimuli, and after his to visit to Claus in Vienna, he published his findings in 1883 (Metchnikov 1883: Fig. 2.10b). Metchnkov writes in "Lectures on the Comparative Pathology of Inflammation" that the same effect was observed with either a rose thorn, a sea urchin spine or a delicate glass rod; and that the mass of phagocytes was often visible to the naked eye. Furthermore, if the thorn was first soaked in carmine or indigo before insertion, these coloured particles "were eagerly devoured by the mesodermic phagocytes". Not only did Metchnikov devote the next 25 years of his life to this, by ingenious experiments, publications and arguments, but many others joined him.

Metchinov's choice of the starfish larva, which he tells us in his "Lectures on the Comparative Pathology of Inflammation" was a bipinnaria**,** the first stage in the larval development of the common starfish *Asterias and is as "transparent as water"* (Fig. 2.10c, d). This optical transparency was the key to the success and foreshadows, by more than a 100 years, the current use of Zebra fish as a model organism for studying inflammation and wound healing *in vivo*.

While Metchinov's starfish experiment was of obvious importance, there were a number of detractors. One of the major counter-arguments Metchnikov faced was from the "old guard" who still hung on to the theory of "spontaneous generation" of germs. The belief and evidence for this came from looking at the microscopic life which seemed to appear spontaneously in water left in contain with hay and other infusorions. When phagocytic cells were seen at sites of infection, such as pus and wounds, it was "obvious" to the old guard that these cells were "bad guys" and were simply carrying the spontaneously generated germs to the wound site. Olga Metchnikov says that the famous physician and cell biologist Rudolf Virchow warned Metchnkov of this as follows:

> *Metchnikoff was also greatly encouraged by Virchow, who happened to pass through Messina and came to see his preparations and his experiments, which seemed to him conclusive. However, Virchow advised him to proceed with the greatest prudence in their interpretation, as, he said, the theory of inflammation admitted in contemporary medicine was exactly contrary to Metchnikoff's. It was believed that the leucocytes, far from destroying microbes, spread them by carrying them and by forming a medium favourable to their growth.*

Metchnikov's counter argument to those who raised this was two-pronged; firstly that they should do experiments to refute his idea; Metchnikov pointed out that Pasteur's experiments had shown that germs do not "spontaneously generate"; secondly that they should consider Darwinian logic. Metchnikov (and others) could demonstrate the presence of phagocytic cells in all phyla and species, including those where they were no longer needed for digestion of food. If there were only a harmful role for phagocytyes (ie carrying germs to sites of injury), Natural Selection would exert its pressure and those animals which had no germ-carrying phagocytes would be more fit to survive, and so animals with germ-carrying phagocytes would dwindle away, to be replaced by animals with no germ-carrying phagocytes. It was easily verifiable evidence, that all animals retained motile phagocytes. This point was especially strong to the supporters of Darwin, pointing to a crucial and beneficial role for these cells. Far from phagocytes being "bad" and dangerous, these cells were clearly *essential* for health.

In 1888, Pasteur had given him a laboratory and an appointment in the newly built Pasteur Institute in Paris, and Metchnikov finally left Odessa for good. This was where he undertook many important experiments and published key papers (eg Metchnikov 1889) and books, including two volumes on the comparative pathology of inflammation (1892), and *L'Immunité dans les Maladies Infectieuses (1901), tranlsted into English as* (Metchnikoff 1905). He was given many awards, most notably the Nobel Prize for Physiology or Medicine in 1908, which he shared with Paul Ehrlich. He spent the rest of his life at the Pasteur Institute in Paris and was so attached to the place that when he died in 1916, at his request, his ashes "were enclosed within an urn and placed in the library of the Pasteur Institute" (where they still remain).

The implications of Metchnikov's insight and work are more the subject of immunology than phagocytosis. However, those who are interested

about the impact of Metchnikov's thorn in the starfish experiment and how it led to the understanding of the innate immune system can read, for example, (Nathan 2008, Cavaillon 2011; Merien 2016; Silverstein 2011; Teti et al. 2016; Nauseef 2014). Perhaps the profound implications of Metchnikov's simple "Eureka" experiment can be summarised best by Olga Metchnikov in her book:

> *This very simple experiment struck Metchnikoff by its intimate similarity with the phenomenon which takes place in the formation of pus, the diapedesis of inflammation in man and the higher animals. The white blood corpuscles, or leucocytes, which constitute pus, are mobile mesodermic cells. But, while with higher animals the phenomenon is complicated by the existence of blood-vessels and a nervous system, in a star-fish larva, devoid of those organs, the same phenomenon is reduced to the accumulation of mobile cells around the splinter. This proves that the essence of inflammation consists in the reaction of the mobile cells, whilst vascular and nervous intervention has but a secondary significance. Therefore, if the phenomenon is considered in its simplest expression, inflammation is merely a reaction of the mesodermic cells against an external agent. Metchnikoff then reasoned as follows: In man, microbes are usually the cause which provokes inflammation; therefore it is against those intruders that the mobile mesodermic cells have to strive. These mobile cells must destroy the microbes by digesting them and thus bring about a cure.*

Inside the Phagocyte

From the start of the twentieth century, the history of phagocytosis accelerates and becomes "modernised" and becomes more familiar to modern science quite quickly. I have therefore chosen just two discoveries, the importance of which still resonating at the start of the twenty-first century.

Phagosomal pH

The first is the pH of the phagosome which, though not strictly part of the process of phagocytosis itself, it is a post-phagocytic event clearly triggered by phagocytosis. Following Greichen in the late 1700's, who first used coloured particles to convince himself that external particle really did end up inside living cells, this became a fairly routine approach. But it was not until 1847, that Rustizky hit on the idea that if the colour of the particle were sensitive to a chemical change, information could be gleaned. He was interested in bone resorption by "giant cells" and reasoned that, since bone is dissolved by acid, the giant cell must generate acid to absorb the bone. He therefore fed litmus particles to his cells and watched. He explains what happened in his 1847 paper,

> *In this experiment I brewed quite neutral litmus powder in the loveliest manner of Baron Dr. Baumann. The giant cells were lifted out of the bone and immediately transfer it to the object-bearer, in which was already saline solution with the litmus additive. Very soon the litmus disappeared and the giant cells became purple in colour, especially at their centres. These objects usually looked blue, but they appeared so coloured under the Gundlaeh microscope, ... especially near the nuclei of the cells, where they even had a yellowish tone.*

Rustizky may have felt that he had proved his point, as the colour change of litmus from blue to red indicated a pH change towards acidic, and yellow was even more acidic. It is not clear from Rustizky's report that he knew the litmus particles were inside vacuoles, although clearly they were. However, thirty years later, Theodor Englemann repeated this experiment on other cell types which phagocytosed particulates. Engelmann was interested in "protoplasm" and especially its contractile properties. As part of this study he used litmus particles and he reports in his paper in 1879:

> *In life, the reaction of protoplasm is generally weakly alkaline or neutral..(but) ... now and then I have seen blue litmus particles change within a few minutes after being taken into the contractile endoplasm of Stylonychia mytilus and S. pustuluta, Paramcecium aurelia, and Amoeba diffluens, to a red colour and remain so.*

Engelmann makes no comment on this observation, presumably because it was peripheral to his main study. Helpfully, however, the English translator of this paper in 1884, A.G. Bourne, adds a footnote saying that "*this is possibly due to an acid secreted in an attempt, to digest the particles*."

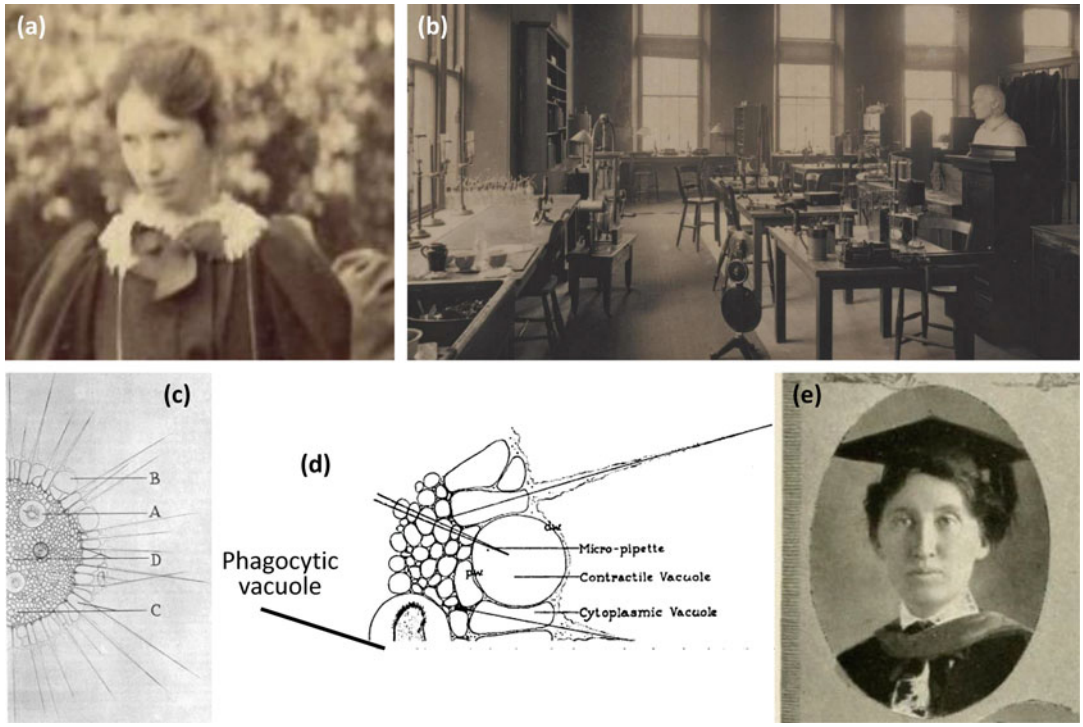

Fig. 2.11 Pioneers of intraphagosomal pH. (**a**) A photo of Marian Greenwood at Newnham College 1896 when she was Director of the Balfour Laboratory.This image was taken from a group photo of Newnham staff standing on the grass behind the Balfour Labs. (**b**) The interior of the Balfour Biological Laboratory for Women showing the simple microscopes on the benches by the windows that Greenwood may have used. The whole lab is watched over by the sightless eyes of the bust of Francis Balfour (the lab's founder). (**c**) Ruth B, Howland's drawing of the heliozoan showing the spines, and internal organelles, with the targets for microinjection indicated, A = phagocytic vacuole. (**d**) shows the micropipette in position (in this case the in contractile vacuole) taken from Howland (Howland 1930). (**e**) Ruth B. Howland at Sweet Briar College in 1919, taken from "Briar Patch", the College Year book. I am grateful to The Principal and Fellows, Newnham College, Cambridge for permission to use the rare photographs of Marion Greenwood and the Balfour Laboratory

The concept of probing the intracellular, or in this case intraphagosomal, environment with an optical probe, whose signal can be detected microscopically outside the cell is one that is still used today. But the early researchers who used the litmus test to look at phagosomal pH soon discovered some surprising things. Firstly, not every cell type or even every cell showed the acid change. An early adopter of the methodology, Greenwood (Fig. 2.11a) reports in her papers of 1886 and 1887 that the litmus indicator did not produce convincing evidence of an acid intravacuolar reaction (Fig. 2.11c). Marion Greenwood (1862–1932) was an English cytologist, who undertook her microscopical work at Newnham College Cambridge, UK, where she was one of the first women to do independent research in Cambridge University. She also directed the newly established Balfour Biological Laboratory for Women (Fig. 2.11a, b). In 1895 she was the first woman to speak about her work at a Royal Society meeting. From her equivocal work with litmus, she suggested that as litmus particles were not "food", they did not necessarily stimulate the digestive response and ultimately concluded that the cytoplasmic secretion into the vacuole was 'probably not acid'. In the following year, Meissner (1888), used a different indicator, alkanet (*Alkanna tinctoria*), which is blue in strong alkali, becoming increasingly "crimson" as acidity increases (blue at pH 10; purple at 8.8 and red at pH 6.1). Meissner showed that *"Amoeba*

princeps" took up globules of olive oil dyed red with *"tincture of alkanna"* and reported that this dye gave a clear demonstration that "the digestive vacuoles of lower organisms" were acidic. A year after Meissner's paper, Metchnikoff (1889) too looked at the litmus test. He states that it is accepted that protozoa "secrete around the object they englobe, an amount of acid sufficient to turn blue litmus red". However, when looking at the important mesodermal phagocytes, he reports his own experience as follows

> *I placed a few grains of blue litmus in the water containing young spongilla … (which)… were soon englobed by the sponges and were found to be taken up chiefly by the mesodermic phagocytes. The litmus however did not change colour, even after a prolonged stay in the cells.*

As this seems to be contrary to what was reported in other cell types, Metchnikov unusually cites an earlier report to support his observation. He refers to Krukenberg (1882) who gives biochemical evidence that tryptic digestion by sponge extracts occurred without the need for acid. When Metchnokov repeats the litmus test on his phagocytic leukocytes, he also reports in his "*Lectures"* a disappointing effect

> *In a large number of experiments that I have made on the absorption of granules of blue litmus by leucocytes, I have seen the colour change to red in only a few exceptional cases*

Presumably, Metchnikov was able to distinguish between litmus particles which were adherent to the phagocyte surface and litmus particles within phagosomes. Unfortunately, he does not follow up the "exceptional cases" when he saw the litmus turn red. However, in the Annals of the Pasteur Institute, he reports one such exception:

> Although this study is still far from finished, it has already shown me the existence of facts analogous to those which have been reported for the Protozona. Thus, after having cut the end of the tail of the newt larvae, Triton taeniatus, and rubbed the wound with a blue litmus powder, I was able to observe that the incoming uninuclear leucocytes partly change litmus grains which are englobed inside them, bright red. In some of these macrophages there was, next to a red litmus grain, a vacuole filled with blue granules of the same substance, which proves that the production of intracellular acidic juice can be localized in a restricted part of the cell.

The observation that the litmus particles became red in some phagosomes but not others led Metchnikov to a remarkable conclusion, namely that chemical changes can be restricted to part of the cell, a topic which (as sub-cellular localisation) is still under discussion. Netchaeff (1891) thought that in the cases when Methchnikov saw the litmus colour change, it was "simply an optical illusion". Netchaeff never saw such a colour change in his own "observations on the fate of limus granules in the interior of leukocytes". Metchnokov countered that Netcahaeff can never have looked at Protozoa, where the pH change was obvious and that Metchnikov's own research "*over a series of years, left him in no doubt as to the reality of the colour change of the litmus*".

However Metchnikov, in his "Lectures" concludes ultimately that "*digestion is carried out in leukocytes in neutral or alkalai medium, as in the case of phagocytes from the sponges*", leaving the confusion of whether phagosome pH changes occur or not and whether they are important or not.

The litmus colour change, as it appears to the eye, is almost a threshold effect and therefore difficult to follow dynamically. However, it was realised that the pH changes were dynamic. As early as 1891, Le Dante reported 'seeing the slow secretion of an acid … the acidity is progressive, as if it was caused by a secretion' (Le Dantec 1890) and Greenwood & Saunders, 1894 and Saint-Hilaire, 1904 also reported that the acid change was transient. A consensus was thus forming that the pH change had two phases, the first, an acid phase, the second, alkaline. However, when phenol red was used as a water phase pH indicator, its gradual colour change over the crucial range (from yellow pH 6.8 to red pH 8.2), reported that in paramecium, there was an even earlier pH phase which was alkaline (Shipley and De Garis 1925). By 1927, Shapiro had reported some exact values for the pH changes by using a range of pH indicators, neutral red, congo red and phenol red. All three indicators detected the acid phase in Paramecium, with values between pH 4.0–4.8 in the three organism tested (ie Paramecium, Vorticella and Stylony-

chia); with paramecium alone showing the initial small alkaline phase. Ruth B. Howland (1928) made a major step forward. After undertaking Ph.B. and Ph.M. degrees at Syracuse University and research as a graduate student at the Marine Biological Labs, Woods Hole and at Yale University, Howland (Fig. 2.11e) became Professor of Biology at Sweet Briar College, at an all-female college on the foothills of the Blue Ridge Mountains in Virginia. From here, she continued her research by making links with outstanding scientists. She decided to use a series of pH indicators, which were truly H^+ ion indicators with known pKa values, namely phenol red, bromothymol blue, bromocresol purple, bromocresol green and bromophenol blue. This in itself would be a step forward, but she wanted to microinject these indicators into phagocytic vacuoles within the cell (a heliozoan, actinosphaerium eichhorni). At this time, microinjection was in its infancy, and the success of this approach was a technical triumph. By making long and thin (and sharp) micropipettes, Howland caused the minimum of injury to the cell despite having to pass the micropipette through "the cytoplasm to reach the deep-lying vacuoles" (Fig. 2.11d). At that time, Howland was based in New York at Washington Square College and Cornell University Medical College, New York, near Chambers and his pioneering microinjection approach. Howland had published a paper with the "star pupil" of Chambers, Herbert Pollack (see more later) a year earlier (Howland and Pollack 1927a, b), so it is probable that the work on phagosomal pH could not have been done with Pollack's assistance. Without modern inverted microscopes, Howland used "micropipettes . . . bent upward at right angles and raised from below into the gastric vacuoles". She states that "*a striking feature of these injections is the complete localization of the dye in the injected vacuole. This permits remarkably accurate color determinations, since there is no loss of injection fluid by outward diffusion*". She then compared the colour of the indicator within the vacuole with that in the standard tubes (ie indicator at different pH values) by placing the standard tubes between the source of illumination and the mirror to find the pH which matched the colour. This colour matching technique was also reported by Chambers and Pollack in 1927, which Howland acknowledges this in her paper. Using heliozoa, Howland measured the early phagosome (closed but with the prey still moving) at pH 6.6–6.9 with a decrease over the next 5–10 min reaching a final minimum of pH 4.3 ± 0.1. As the pH in the vacuole fell, the lethality increased, judging by the lack of movement of the prey. To test whether this fall in pH was a consequence of the death of the prey, she "*crushed and tore*" large ciliates and rotifers in microdroplets of bromocresol green and found that this alone caused a decrease in pH to 5.5 ± 0.1. This "usual acid of injury", as she called it, was only 1/10th the H^+ ion concentration that she found in the phagosome, and so she concluded that acid was secreted into the vacuoles by the living cytoplasm of the heliozoan.

The topic of intraphagosomal pH continues to be discussed and research undertaken 150 years after the first reports. It is surprising that similar or even the same techniques are still used today. For example, Geisow et al. (1981) produced an important paper entitled "Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy", in which the title suggests they used fluorescence intensity (rather than colour) as the indicator of phagosomal pH. The conclusions they reached for macrophage phagosomes were similar to those of the 1920s, namely that "*the pH in new phagosomes was transiently driven alkaline*", just as had been reported in paramecium (but not other cells) over 100 years before. Their re-discovery was, surprisingly, based on some familiar older indicators which they report in a way that would be familiar to Greenwood, and Howland in the 1920s (and before) reporting that "*neutral red yeasts seen entering macrophages turned from red to a pale yellow and returned to a brilliant red within 1 min* " and "*bromothymol blue yeasts (yellow-green in the BSS) turned blue after entry, then green, and then yellow*". From this they conclude "that the phagosomal pH is first increased from that of the external medium (to at least pH 7.5) and then within 5 min is reduced to a pH <6.5". Since then, advances in the design

of pH fluorescent probes and the technology required to acquire ratiometric spatial data have made intraphagosomal pH measurements more secure (Nunes et al. 2015; Canton and Grinstein 2017) and the molecular details of the controlling factors for the phagosomal pH are now being established (eg Jankowski et al. 2002).

Ca^{2+} Ions and Phagocytosis

From the end of the 1800s to the first 20 years of the 1900s, there was a major increase in Universities, professional scientists. There was also an advance in technology and understanding. During this time, the study of phagocytosis began to probe the cellular mechanisms behind this event. Phagocytologists at this time wondered what factors in the extracellular environment were important for phagocytosis, including Ca^{2+}. However, there were conflicting reports that extracellular Ca^{2+} inhibited or enhanced phagocytosis, and that Mg^{2+} and other ions had similar effects. It is surprising that many of the early studies had no regard for the effects of osmolarity, and simply by adding large amounts of Ca^{2+} or Mg^{2+} or other ions (often in the 100 mM (M/8) range) phagocytosis was inhibited by osmotic shrinkage of the cells. Also no test system used by these early investigators were identical. Also there was the dawning realisation that factors in serum (when looking at blood phagocytes) opsonised some particulate stimuli, and that many reports simply reflected the requirement for divalent ions for binding of the stimulus. Thus, these studies were not really looking at phagocytosis itself, since phagocytosis was never initiated. The data at this period is therefore a muddle. However, there was a beam of light from Sidney Ringer (1835–1910) who showed that in order to maintain muscle contracts, it was crucial that Ca^{2+} ions were in the perfusion solution. Following this, Hartog Jacob Hamburger (1859 – 1924) looked a factors affecting phagocytosis, including Ca^{2+}. He clearly knew of the work of Ringer and even came up with a rival to "Ringer's solution" namely "Hamburger's solution". Hamburger (Fig. 2.12a), who was Dutch, and studied chemistry at Utrecht University from where he received a doctorate in 1883 and in 1901 became professor of physiology at University of Groningen. It was here that he published a book entitled *Osmotischer Druck und Ionenlehre in den medecinischen Wissenschaften* ("Osmotic pressure and ion science in the medical sciences"). With this background, Hamburger would not make the osmotic mistakes of previous studies. He also hit on a robust test system for studying phagocytosis. While others were trying to reproduce in the laboratory what happen in the body (or elsewhere) even if unaware of all the complexities, Hamburger wanted an experimental system which, while artificial, was controllable and chose carbon as the phagocytic target. Hamburger could see the advantages, (i) "carbon" particles could be produced as a standard stimulus (Fenn later showed that neutrophils had a preference for carbon particles over some other particulates (Fenn 1923: Fig. 2.12b), (ii) could be visualised easily in living cells under the microscope and (iii) Hamburger thought carbon was not influenced by any unidentified factors from serum, although Fenn (Fenn 1921) later reported that in his cells, serum was required. In his first report in 1910, to the Royal Netherlands Academy of Arts and Sciences (KNAW), Hamburger reports a remarkably set of results (Hamburger 1910). He firstly showed that "small amounts of calcium" had effects on phagocytosis under normal experimental conditions. However, if the leucocytes were left in a Ca^{2+} free medium (NaCl saline) for 24 hours, phagocytosis was almost non-existent (less than 3% of cells internalised carbon particles). This then was the experimental condition that Hamburger needed to test the effect of ionic replacement on phagocytosis. He reports that adding Mg^{2+}, Sr^{2+} and Ba^{2+} to the medium had no effect on the ability of the cells to phagocytose, but that adding Ca^{2+} to the medium restored phagocytosis to its normal level (50%)

> These experiments show that when the phagocytes, by being exposed a long time to NaCI 0.9 %, have almost entirely lost their power, they cannot be revived by barium (or magnesium or strontium). An isosmotic quantity of calcium however, produces this effect (ie revival of phagocytosis) in a very marked degree

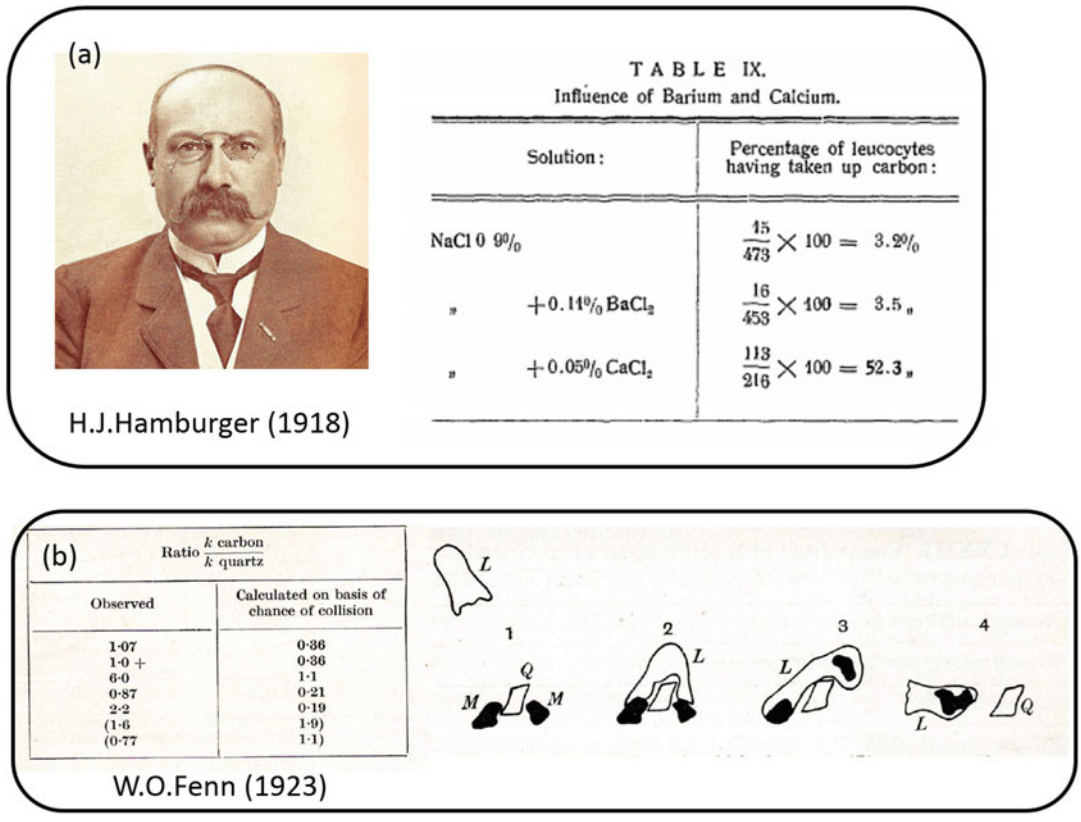

Fig. 2.12 H.J. Hamburger: Ca^{2+} ions and phagocytosis (part I). (**a**) Shows a photo of H.J. Hamburger in 1918 together with his crucial data showing the unique role of Ca^{2+} in restoring phagocytosis. This data (or slight variants of it) were shown in papers published by Hamburger in Nature, Brit Med J. and Royal Netherlands Academy of Arts and Sciences (Amsterdam) Proceedings (Hamburger 1910, 1915, 1916). (**b**) Shows some results from W. O. Fenn, showing the preferential uptake of carbon particles compared to silica and a leucocyte stretching" around a particle of silica (S) to phagocytose two particles of manganese oxide (M). The whole sequence was 120 s (Fenn 1922)

The original data for this statement in a series of tables, the key one taken from Hamburger's first paper is shown here in Fig. 2.12a. The amount of $CaCl_2$ added (0.05%) which totally restored phagocytosis (if the calcium chloride crystals were hexahydrate $CaCl_2.6H_2O$ as is usual) was 2.3 mM Ca^{2+}. In other experiments, restoration was seen with Ca^{2+} as low as 0.23 mM. These concentrations are within the usual mammalian physiological range or less than those in other environments (eg in artificial sea water Ca^{2+}= 10 mM). Hamburger's results were clearly physiologically significant and suddenly there was clarity. Calcium (or rather Ca^{2+}ions) was a key element in phagocytosis. Hamburger followed this paper with confirmation (Hamburger and de Haan 1910) and rightly became famous for this discovery. He reported the same observations in a number of papers, including verbatim copies in Nature (Hamburger 1915) and in the British Medical Journal (Hamburger 1916). Without knowing the importance of his finding, McJunkin (1918) also showed that simply by adding citrate (15 mg/ml ie c.70 mM), phagocytosis by neutrophils was prevented. This Ca^{2+} chelator at that concentration would have significantly depleting effect on the free Ca^{2+} in the system and seems, therefore, to be a confirmation of the reduction in phagocytosis which Hamburger saw in the absence of extracellular Ca^{2+}.

Hambuger, however, was unsure of the mechanism by which Ca^{2+} exerted this effect, but cleverly drew a conclusion which today, we now know to be true

> We might be inclined to attribute the increase (in phagocytic ability) ... as a consequence of the electric charge, caused by the entering of a number of bi-valent calcium ions. This explanation however can hardly be the correct one here, for experiments show that other bi-valent cations – namely barium, strontium, magnesium -do not augment the amoeboid motion. It must be assumed then, that the action of calcium in this case, is based upon an *unknown specific biochemical property* of this metal

Within 20 years, a major step forward in understanding that changes cytosolic Ca^{2+} was the answer, was made by the ingenious work of the young Herbert Pollack (1906–1990). The Washington Post tells us in his obituary that during World War II, Pollack was a colonel in the Army Medical Corps, serving in Europe as the U.S. representative on the Inter-Allied Commission for the Study of Prison and Concentration Camps. He was decorated with the Bronze Star, the Purple Heart and the Army Commendation Medal. In the Korean War, Pollack visited the war zone for the surgeon general of the Army to review the medical evacuation system and that he also received an Outstanding Civilian Service Medal from the Army for work in connection with the threat of malaria in Vietnam and another for work dealing with high altitude physiology at the time of the Chinese invasion of Tibet. What the obituary does not tell us is that before he began his private medical practice in New York City in 1934, he did some amazing ground-breaking cell biological experiments.

This was when Pollack was a student of Robert Chambers, who was a pioneer of microinjection techniques which he called micrurgery (Chambers 1921, 1922). It was to Chambers' laboratory in New York that Ruth Howland came to microinject phagosomes (see above) and published a paper with Pollack (Howland and Pollack 1927a, b). She was obviously skilled at delicate microinjection because in Pollack's crucial paper, she is thanked (in a foot-note) for her "help". Herbert Pollack was only 20 years old when in Chambers laboratory (Fig. 2.13a), but he produced perhaps the key paper. Although his paper was part of a numbered series from Chambers' laboratory, Pollack is the single author. However, before we see Pollack's major contribution, it is important to see it in context. Chambers' laboratory was cutting edge having developed new microinjection technique (Fig. 2.13b–d) for investigating properties of "living protoplasm". He included line drawings of cells as had been done previously, but there are also photographs taken through the microscope of some of their experiments. This, of course, was not a first. In 1917, a "cinematographic recording" of phagocytosis was presented by Comandon (1917) to the "The Society of Biology and its subsidiaries" (La Societe de Biologie et de ses filiales). I am not sure of the impact it had, but even today an AVI.file of an experiment involving phagocytosis is often appreciated by the audience. But clearly Chambers was in the vanguard of modern science. Chambers and Reznikoff (1926) explored the effect of microinjecting ions into the cell, especially amoeba. They report that injecting Ca^{2+} caused an immediate "solidification of the cytoplasm" which results in it a pinching off. This would seem to be a pathological response, caused by extremely high cytosolic Ca^{2+} such as M/13 (77 mM Ca^{2+}), but these effect persisted at Ca^{2+} injection concentrations down to M/104 (9.6 mM Ca^{2+}). Below this concentration, they report

Fig. 2.13 (continued) alizarin and saw the first evidence of localised Ca^{2+}signalling within amoeba and its relationship to pseudopod progression. The photo was taken from the US National Library of Medicine Collection (https://collections.nlm.nih.gov/catalog/nlm:nlmuid-101439620-img) with permission of the copyright holder. (**b**) Shows the method for fabrication of the micropipette using a Bunsen burner, and the shapes they produced (from Chambers 1921, 1922). (**c**) Shows a close-up of the microscope/injector in front of Pollack and (**d**) shows a higher resolution image of the detailed assembly of pipes leading to the moveable pipette holder beneath the microscope stage as shown in Chambers (1921, 1922)

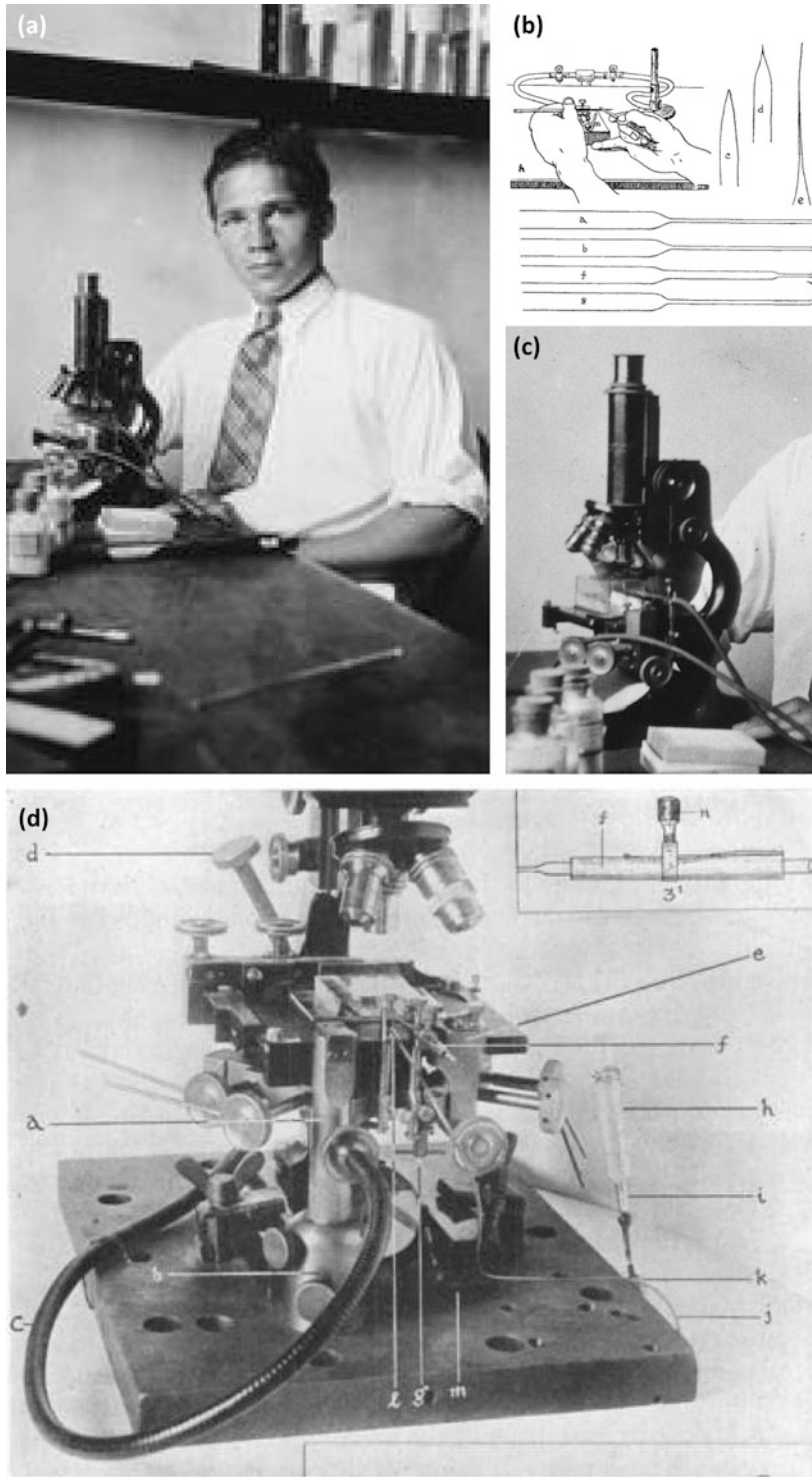

Fig. 2.13 Herbert Pollack: Ca^{2+} ions and phagocytosis (part 2). (**a**) Shows a photo of Pollack when a medical student (about 20 years old) working in Chambers' lab. In front of him is the microscope with microinjection equipment developed by Chambers' group. It was with this equipment that Pollack microinjected amoeba with

The dilution of M/208 (ie 4.8 mM Ca^{2+}) appears to be the critical strength at which the pinching off is either delayed from 2 to 10 minutes or is never completed. In the latter case, the involved region is ultimately resorbed. With the dilution of M/416 (ie 2.4 mM Ca^{2+}) no pinching off is even attempted and the ameba reacts as if it had been injected with water alone.

We may assume that injection of the lowest concentration of Ca^{2+}, M/416, did not elevate the level of cytosolic Ca^{2+} significantly (ie it is near the null point, now known to be about 100nM). If the injectate was approximately 0.25 the volume of the cell (as seemed to be the standard procedure for the Chambers' lab.), then the total Ca^{2+} added would elevate the cytosol by 0.6 mM (ie 2.4/4 mM). Since there was no cellular response, the free cytosolic Ca^{2+} the injection generated was at most only 100nM, allowing us to estimate the cytosolic buffering capacity for Ca^{2+} to be about 6000:1. This is not an unreasonable figure and in line with many estimates made today for the "slow Ca^{2+} buffering" component (ie from 1000 to 10,000:1). With this estimate, the M/208 Ca^{2+} injection, giving only a weak or zero response, would elevate cytosolic Ca^{2+} by 200nM; M/104, M/52 and M/26 would elevate cytosol Ca^{2+} to 400 nM, 800 nM and 1.6 μM respectively, and trigger a robust responses. These cytosol Ca^{2+} concentrations are, of course, only "guestimates" because the restricted diffusion of cytosolic Ca^{2+} would the cause a localisation of injected Ca^{2+}; and so these estimates represent minimum vales. It is, however, interesting that these are all in the range of cytosolic Ca^{2+} increases that we now know occur physiologically. It is thus possible that these represent feasibly physiological responses. This may, thus, be the first time that the concentration of cytosolic Ca^{2+} (and the first time its dynamic range for stimulation) had been estimated in phagocytes.

It is possible that Pollack was aware of the possibility that this cytosolic gelation was important in amoeba and may have already observed reversible gelation near the contractile vacuole, which was thought to be essential for vacuole contraction (Howland and Pollack 1927a, b). But this is not why phagocytologists should be interested. Instead, it is because, pseudopodia formation, whether in amoeba or other phagocytes, is a key part of phagocytosis. Howland and Pollack (1927a) noted that cytoplasmic gelation occurred "in the greater percentage of cases . . . in the posterior portion of the ameba" and Chambers and Reznikoff (1926) reported that microinjections of "$CaCl_2$ . . . solidify the internal protoplasm . . . (which) . . . solidification tends to be localized . . . (and) . . . the injection of $CaCl_2$ accelerates movement in the regions not solidified". Pollack must also have been aware that Reznikoff and Chambers (1927) had found that the microinjection of phosphates, carbonates, and sulfates immediately but temporarily prevented amoeba from forming pseudopodia (Reznikoff and Chambers 1927). Pollack noted that these anions form insoluble salts with Ca^{2+} which raised the possibility that their effect on inhibiting pseudopodia formation was via a reduction in cytosolic free Ca^{2+}. In his paper (Pollack 1928), Pollack tested whether the effect was due to it effect of cytosolic Ca^{2+} by microinjecting "*two other organic anions whose calcium salts have relatively low solubility products, viz., tartrate and oxalate.*" It must have been an exciting moment when he saw a similar effect on pseudopodia formation, and that the amoeba injected went through the same "*stages of quiescence, rounding, and pseudomembrane formation*". Pollack reports that "*the ameba could recover from a moderate injection of M/8 solution of sodium potassium tartrate* (ie 125 mM) *or of M/18 solution of sodium oxalate* (ie 56 mM)" usually in a few hours. The "*peudomembrane effect*" (ie the result of quiescence and rounding) was also caused by lower amounts of the Ca^{2+} reducing agents with "*concentrations as low as M/128 of sodium potassium tartrate and M/620 of sodium oxalate* (ie 1.9 mM and 400 μM respectively in the cytosol)". Clearly the formation of insoluble calcium tartrate ($CaC_4H_4O_6$) and calcium oxalate (CaC_2O_4) in the cytosol at these high concentrations, would have a significant and long lasting reducing effect on cytosolic free Ca^{2+} from its initial concentration of 100nM and in suppressing Ca^{2+} signals.

Pollack wrote that the "first effect of the injection of any of the calcium precipitants is

absolute quiescence." This was good evidence for a role for cytosolic Ca^{2+}, but as Pollack was realising that Ca^{2+} inside the amoeba was important for pseudopodia formation, he recognised that he needed an indicator of Ca^{2+} that he could microinject into the cell. His options, at that time, were very limited. However, alizarin, a dye known from antiquity as "madder", precipitates with Ca^{2+} and had recently been reported for use as the basis of the measurement of "small quantities" of Ca^{2+} in blood (Laidlaw and Payne 1922). Pollack intended to use alizarin as an optical indicator to watch a chemical change occur within a living cell in real time. This was an ambitious and ground-breaking experiment, but having injected alizarin, he reports simply:

> The injection of a moderate quantity (1/4 the volume of the ameba) of a saturated aqueous solution of this reagent (ie alizarin reddish brown in color) causes a temporary cessation of movement.

As Pollack had expected (and hoped), alizarin acted as a simple cytosolic Ca^{2+} reducing salt, like oxalate, and pseudopodia formation was inhibited. However, unlike oxalate, Pollack could see the crystal of calcium-alizarin form within the cell. He reports:

> *The ameba rounds up and the larger crystals (of undissolved alizarin) and granules may settle to the bottom. A close examination of the cytoplasm shows fine purplish red granules scattered throughout the cell, and the hyaline cytoplasm itself is diffusely colored pale red.*

He noted that

> *If an ameba is killed during the injections or is torn by the micro needles in a medium containing alizarin, the large crystals normally present in the ameba and some of the coagulum which is produced upon death will also take on the purplish red color characteristic of calcium alizarinate.*

Thus exposure to the high Ca^{2+} of the extracellular environment was detected as "*purplish red calcium alizarinate crystals*". Pollack then records, as follows, the key observation:

> *If the ameba tries to pull forth a pseudopod as evidenced by a slight lifting of the membrane, a shower of these purplish red granules are seen to appear in this area and the pseudopod formation is immediately stopped.*

The "shower of purplish red crystals" which appeared were reporting an elevation of Ca^{2+} in the cytosol. He had seen, for the first time, a localised rise in cytosolic Ca^{2+} associated with the formation of pseudopodia. This was not a chance observation, as some of the early work of phagocytosis was, but instead it was the result of a careful train of deduction and careful experiments.

It is interesting that Pollack says that the inhibited amoeba "tries to pull forth a pseudopodia" as it is difficult when observing phagocytosis or other cell movements not to feel the cell is "trying" to do something. Of course, what Pollack really witnessed was the protrusion of a pseudopodium in response to a spontaneous local elevation in cytosolic Ca^{2+}, which was then aborted by the precipitating effect of the alizarin. The localised precipitation of calcium-alizarin as a "shower of red crystals" quenched the Ca^{2+} signal with the result that that pseudopod extension stopped. The sudden appearance of the shower of red crystals, must have been the result of an elevation of cytosolic Ca^{2+} to a level above the solubility limit for calcium–alizarinate as Pollack reasoned:

> *The quiescence which is induced after an injection of alizarin may be due to a removal of calcium of the protoplasm from the sphere of action.*

In order to test this idea, Pollack designed an additional set of experiments aimed at reversing the effect by additional Ca^{2+}. He writes:

> *When an ameba which has previously been injected with alizarin is injected with an M/208 calcium chloride solution, active flowing movements appear almost immediately which subside in a very short time.*

The level of Ca^{2+} injection was estimated earlier to give a rise in cytosolic free $Ca^{2+}$of 200 nM at equilibrium, with higher concentrations locally and at earlier times. It is interesting that Pollack does not mention seeing red crystal forming as the Ca^{2+} injection is done, so it could be that the local Ca^{2+} change during pseudopod extension was higher than the effect of microinjection (ie 200 nM). Pollack noted that the recovery time depended on the amount of alizarin injected, and

that after Ca^{2+} injection, the cells recovered the ability to form pseudopodia at a faster rate ie *"the time usually required for complete recovery after an alizarin injection is shortened from about 2 to 3 hours to ½ to 1 hour.*"

Pollack's conclusion is one that has many resonances to "Ca^{2+} signallers". In his conclusion, he gives concepts with are still vitally important today eg free Ca^{2+} versus un-ionised total calcium: Ca^{2+} equilibrium in the cytosol: mobilisation of Ca^{2+} reserves (stores?). He writes:

> *The fine, purplish red granules resulting from the injection of the alizarin are, no doubt, the insoluble calcium alizarinate. Recovery of an ameba from such an injection may be explained by the postulate that the free calcium ions in the living ameba are in equilibrium with a reserve supply of unionized calcium. The equilibrium is upset when the free calcium is removed by precipitation or by other means, and the system may possibly react in such a way as to counteract the effect of the change imposed. By mobilization of the calcium from a reserve supply the ameba can therefore gradually resume its normal activity.*

These conclusions were reached by a 20 year old student nearly 100 years ago and yet are still largely accepted today. It was not until 1980 that his observations were essentially confirmed by Taylor et al. (1980) who microinjected amoeba with the chemiluminescent Ca^{2+} indicator aequorin and rediscovered the Ca^{2+} changes which accompany pseudopod formation. Pollack was clearly far advanced not only being the first to detect a change in cytosolic Ca^{2+}, but the first to relate this to a physiological event (pseudopod formation). More than this, Pollack was the first to see a dynamic change of any physiological chemistry within any living cell; and so conceptually open a whole new field of understanding.

Conclusion

As so, as this brief history of phagocytosis draws to a close, there are a few conclusions that may be drawn. The phenomenon of phagocytosis, which appeared, when it was thought that animalcules were simply very small animals, and had the same instincts and behaviours. When hungry, they eat. When they eat, they swallow etc. There seemed no need to ask how the very small animal could swallow etc. Now, we think of cells as a well-organised collection of molecules and ions and that phagocytosis is an "emergent" phenomenon. The components of the molecular/ionic ensemble are now known and the interactions between them are increasingly understood. The more we know, the more complicated it seems. Yet paradoxically, phagocytosis must be one of the most primitive cell activities; being responsible for nutrition and probably key steps in evolution of eukaryote with the inclusion of organelles, especially mitochondria, which probably originated as symbiotic bacterium which had been phagocytosed. Primitive, often implies simple. It is possible that there is a form of simple phagocytosis and that thus has been overlain by modifiers and back-up systems which we are now faced with unravelling. In the same way the C.Elegans a simple (and primitive) organism, has led to a number of discoveries, perhaps a primitive cell displaying the "essence" of phagocytosis without the accrued overlay of complexity may be useful. The history of phagocytosis research has been in the reverse direction. So we now understand more and more of the complexities, without yet understanding the basics. Surely, since phagocytosis has been observed for hundreds of years, and with an accumulated useful knowledge base of many decades and with technology progressing exponentially over this time, fully understanding phagocytosis in the near future is an achievable objective.

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