The *Dark Art* of Blood Cultures

EDITED BY Wm. Michael Dunne, Jr.
and Carey-Ann D. Burnham

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To Linda and Ryley for keeping us sane.

Editors' Disclaimer

While references are used extensively to support observations, published data, clinical trials, and laboratory comparisons of blood culture systems, culture media, protocols and procedures, etc., it is inevitable that personal opinions and small biases might have unintentionally snuck in to the final copy. The editors were very attuned and sensitive to this possibility and made every attempt to avoid blatant conflicts of interest without interrupting the independence of the authors. All of the individuals and commercial entities that have contributed to the advancement of the science of blood cultures have had a significant influence on the field and we thank them all.

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Preface

Many years ago, we began kicking around the possibility of devoting an entire text to the subject of blood culture technology and the continuing evolution that has occurred over the past decades (and centuries if you start with historical perspective). As with any idea, there is often an energy of activation to get the project underway but once that occurred, we were able to gain the support of those involved.

This project was a team effort, and each of the chapters was authored and/or coauthored by the fellows of the Medical and Public Health Microbiology training program established at Washington University School of Medicine and Barnes-Jewish Hospital in 2001. We are equally proud of each of the fellows, and any proceeds generated from this publication will be funneled back into the training program to help keep it alive. We are also thankful for those contributors who worked with former or current fellows to generate some of the chapters presented in the text. The title and cover we selected for this book underscores the slightly warped sense of humor we have integrated into the training program over the years, and even today, there are a number of inside jokes shared by this family as it continues to grow. In that vein (there's the first pun), a good microbiologist who recovers *Eikenella corrodens* from a blood culture will always ask about bite marks. It is our hope that this compendium of all things blood culture will also continue to expand with future editions and will always include graduates of the Washington University program both before and after they leave to direct clinical microbiology laboratories of their own. We might have not provided a complete evaluation of all the technologies that have come and gone over the years, but we'll stick with it (second pun).

Wm. Michael Dunne, Jr.
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About the Editors

Wm. Michael Dunne, Jr., PhD, is currently a senior research fellow for bioMérieux, Inc. in Durham, North Carolina. Prior to joining bioMérieux, he was Medical Director of the Diagnostic Microbiology Laboratory at Barnes-Jewish Hospital and Professor of Pathology and Immunology, Molecular Microbiology, Pediatrics, and Medicine at Washington University School of Medicine in St. Louis from 2000–2011 and remains on the faculty there. During that time, he established an internationally-recognized training program in medical and public health microbiology, which is still active under the direction of Carey-Ann Burnham. He has previously served as Medical Director of Microbiology Laboratories at Henry Ford Hospital (Detroit, Michigan), Texas Children's Hospital (Baylor College of Medicine, Houston, Texas) and Children's Hospital of Wisconsin (Medical College of Wisconsin, Milwaukee, Wisconsin, where he had received his PhD in 1982). Dr. Dunne is a Diplomate of the American Board of Medical Microbiology and a Fellow of the American Academy of Microbiology, the Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the Canadian College of Microbiology. He served as a senior editor of the *Journal of Clinical Microbiology* for ten years and remains on the editorial board. He is also an Adjunct Professor of Pediatrics at Duke University School of Medicine, Durham, North Carolina. He has authored or coauthored over 170 peer-reviewed publications and 10 chapters, in addition to this book.

Carey-Ann D. Burnham, PhD, completed her PhD in Medical Sciences at the University of Alberta in Edmonton, Alberta, Canada and her postdoctoral training in Medical and Public Health Microbiology at Washington University under the direction of Wm. Michael Dunne, Jr. Currently, Burnham is an Associate Professor of Pathology & Immunology, Molecular Microbiology, and Pediatrics at Washington University in St. Louis School of Medicine. Burnham has a keen interest in education and is the Program Director for the Medical and

Public Health Microbiology Fellowship at Washington University, the co-editor of Medical Microbiology Question of the Day, and the section editor of "The Brief Case" for the *Journal of Clinical Microbiology*. Burnham's research program focuses on development of diagnostic assays for infectious diseases and the transmission and epidemiology of antimicrobial resistance in bacteria. Burnham has authored or coauthored more than 100 peer-reviewed publications in addition to numerous invited articles and book chapters.

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1 Historical Perspectives on the Art and Science of Blood Culture

Wm. Michael Dunne, Jr.[1,2,3]

From an historic point of view, one can divide the evolution of blood culture technology into four distinct "archeological" periods that I refer to (tongue in cheek) as the "Manualithic" (pre-1970), the "Bactecene" (1970 to 1990), the "Continuous Monitorassic" (1990 to 2000), and the "Ampliaissance" (post-2000) ages (Fig. 1). Each of these denotes a quantum shift in the use of technology to increase the sensitivity and decrease the time-to-detection of microorganisms in blood. While the rate of improvement in both speed and sensitivity has clearly reached steady state over recent years, and the ultimate goal of directly detecting microorganisms causing bacteremia/fungemia/sepsis from blood has not yet been achieved, progress is most definitely moving forward and the tools being developed for that purpose are impressive. However, before we find out how far we have come, it is best to review where the concept of finding microorganisms in blood began, and some historians would conclude that it started with Athanasius Kircher (Fig. 2).

Kircher was an ordained Jesuit priest whose life nearly spanned the entire 17th century (1602 to 1680). He is referred to as a polymath/savant and his life story reads more like Indiana Jones and the Arc of the Covenant (1). Kircher was a scholar and true renaissance man who became well versed in linguistics, astronomy, magnetism, volcanology, archeology, and acoustics, just to name a few of his scientific hobbies. When the bubonic plague struck Rome in full force in 1656, Kircher extended his credentials to include

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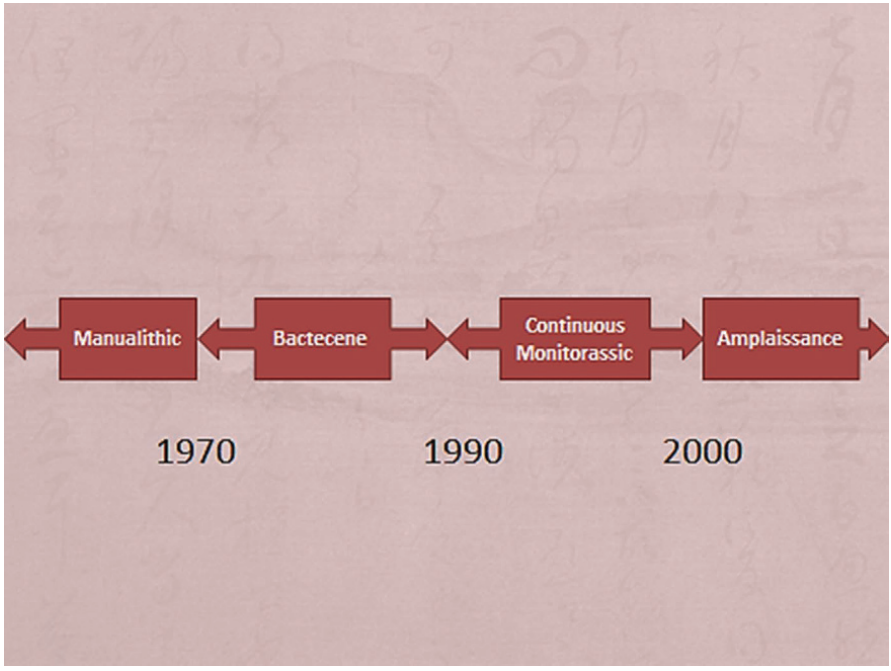


Figure 1 The archeological periods of blood culture evolution.

ad hoc pathologist and infectious diseases researcher (1, 2). Using a primitive microscope, he described observing tiny worms or *vermes* (*contagia animate*) in the "verminous blood of fever patients" and became the first to develop a germ theory of disease, which was published 2 years later (*Scrutinium physico-medicum contagiosæ luis*. Romæ: Typis Mascardi, 1658; references 2, 3). In this treatise, he describes the disease thusly: "*Pestis est flagellum and sagitta Dei ob peccata hominibus immissa*," which loosely translates into "plague is the whip and arrows of an angry God used to punish sinful humans." From a purely microbiological standpoint, two words in this description jump out: pestis and flagellum. Of course, the former became the species name for the causative agent of bubonic plague. However, the latter was inaccurate, because *Yersinia pestis* is nonmotile and lacks flagella. He further stated that "vinegar, milk, and the blood of those sick with fever are full of worms invisible, however, to the unaided eye. That the matter is not otherwise than I have said, the putrid blood of those afflicted by fevers has fully convinced me; I have found it, an hour or so after letting, so crowded with worms as to well high dumbfound me; and I had even been persuaded forthwith that man

Figure 2 Cornelis Bloemaert II, *Portrait of Athanasius Kircher*, 1664/1678. Kircher was a Jesuit priest who launched the notion that certain diseases were caused by microscopic organisms carried in the blood and published his germ theory of disease in 1658 amid the plague epidemic in Rome. The quotation loosely translates to "Plague is the whip and arrows of an angry God used to punish humans." Note the Latin words *pestis* and *flagellum* in the quotation that had unintended contemporary application in current bacteriology. Reprinted from reference 1, with permission.

both alive and dead swarms with numberless but yet invisible worms; and that this may be the meaning of the words of Job: 'I have said to corruption, thou art my father; to the worms, my mother and my sister.'" As fate would have it, the microscope Father Kircher used likely lacked the resolution to visualize *Y. pestis*. What he actually observed remains a mystery, but it was probably clumps of red blood cells (3, 4). However, his speculation that bubonic plague was indeed blood borne and caused by a microorganism was entirely spot on, albeit perhaps a lucky guess (2, 4). He also opined that infectious diseases were not transmitted through the air but rather by person-to-person contact or via another living thing. Kircher also unintentionally invented infection prevention by offering ways of avoiding disease through piety, repentance, and acts of mercy which did not preclude leaving the city, having adequate ventilation, or using odorous substances (2, 4). In fact, it was not until nearly 25 years later that Antonie van Leeuwenhoek had

developed a microscope with sufficient resolution (most likely in combination with some form of dark-field illumination) to observe a microorganism the size of *Y. pestis* (5, 6). So in a sense, Father Kircher was the father of the concept of blood-borne disease.

In a continuation of the theory that blood harbors agents of infection, Professor Jean Antoine Villemin (Fig. 3), a French physician, reported to the Académie de Médecine in 1868 that the injection of blood, sputum, or granulomatous material from animals or humans with tuberculosis into the veins of laboratory animals (rabbits, dogs, cows, etc.) generated lesions in the lungs, bone, and viscera of these animals that were indistinguishable from those produced by active infection (7). Furthermore, Dr. Villemin stressed that the lesions thusly generated via venous inoculation were quite distinct from embolic lesions generated by the inoculation of inert materials such as dust or even pus from other infectious processes. In the strictest sense, Villemin had just described a "biological blood culture," i.e., the use of laboratory animals to recover agents of blood-borne infection.

At the turn of the 20th century, just prior to the outbreak of World War I, the process of culturing blood from septic patients was already a complex

Figure 3 Dr. Jean Antoine Villemin, a French physician, reported to the Académie de Médecine in 1868 that the injection of blood, sputum, or granulomatous material from animals or humans with tuberculosis into the veins of laboratory animals (rabbits, dogs, cows, etc.) generated lesions in the lungs, bone, and viscera of these animals that were indistinguishable from those produced by active infection.

affair beginning with preparation of the skin surface prior to drawing a sample (8). It was recommended that preparation for a blood draw should take place at least an hour prior to collection by using green soap, alcohol, and bichloride of mercury in succession to cleanse the skin as one would do to prepare for surgery. Blood should be collected using a sterilized syringe (30 min of boiling or hot-air chamber) using a linen bandage as a tourniquet. Approximately 10 ml was the target collection volume, and this could be mixed with sterile 2% ammonium oxalate to prevent clotting so that "transfers can be made more leisurely to culture media." In cases where transportation to the laboratory might be delayed, cultures could be prepared at bedside (point-of-care system?). Recommended media combinations consisted of glucose-meat-infusion agar and broth; the former to be melted and combined with blood (0.25 to 1.0 ml) to prepare pour plates. Three flasks containing between 100 and 150 ml of broth were to be inoculated with "varying" volumes of blood, but one flask should be at high dilution. It was further suggested to add powdered calcium carbonate to one flask to ensure a neutral pH, thus supporting the growth of either pneumococci or streptococci that are "sensitive to acid" (do not tell oral streptococci this!) and to "develop and retain their vitality." It was recognized at this point in time that contaminants posed a challenge to interpretation, but one could look at the frequency of isolation (one flask or one plate only) or location (near the edges or on the surface of the pour plate) as an indication of contamination. It was also considered a good idea to regard all staphylococci recovered from blood with suspicion. *Staphylococcus albus* (now *S. epidermidis*/coagulase-negative *Staphylococcus* spp.) should most often be deemed a skin contaminant, whereas the isolation of *Staphylococcus pyogenes aureus* from a single blood culture should be substantiated with a second (8). Patients with suspected typhoid fever prompted an entirely different blood culture strategy that was based on clinical investigations of Coleman and Buxton, Conradi, Schottmueller, and Castellani, as discussed in reference 9. For this method, flasks containing 20 ml of a mixture of ox bile, glycerin, and pepton (peptone) were inoculated with 3 ml of blood and incubated for 18 to 24 h. At that time, samples were subcultured to lactose-litmus agar, and isolated colonies were identified using agglutination or additional biochemical tests. This protocol produced a positivity rate of 89% during the first week of illness, 73% during the second, 60% during the third, 38% during the fourth, and 26% thereafter. In the eighth edition of the *Manual of Bacteriology*, Muir and Ritchie promote two approaches for the culture of blood depending on the organism(s) being sought (10). For enteric bacilli, it was

recommended to mix 5 ml of blood with 10 ml of sterilized ox bile or 50 ml of a 0.5% solution of bile salts in a 1% solution of sodium citrate and incubate the mixture. When examining for other organisms such as streptococci, however, it was suggested that 5 to 10 ml of blood be added to a large volume of liquid medium (e.g., 100 ml)—an interesting observation considering that the recommended blood-to-broth ratio in the absence of neutralizing resins remains the same even today. They also mention the use of trypsin added to broth to neutralize the antibacterial properties of blood and to prevent clotting (Douglas and Colebrook cited in the chapter but no reference provided). The trypsin/broth blood culture medium could be made ahead of time and stored in the ice chest for several weeks.

At the onset of World War I, Mildred Clough, a physician/microbiologist at Johns Hopkins University, first reported on the use of a lysis-centrifugation procedure for the recovery of *Mycobacterium tuberculosis* from the blood of patients with miliary tuberculosis (11). Ten milliliters of blood was lysed with distilled water and the lysate was "centrifugulized" for 1.5 h at high speed. The sediment was then washed over the surface of blood agar slants and incubated. It was noted that colonies of *M. tuberculosis* could sometimes be visualized within 7 to 10 days, which, even by today's standards, would be considered rapid.

One of the more important developments in modern blood culture technology occurred during the 1930s and 1940s and concerned the recognition of "Liquoid" (Hoffman-LaRoche; sodium polyanethol sulfonate) as a potent anticoagulant, anticomplement, and antiphagocytic agent. This substance was found to be useful as a blood transport medium (thus avoiding complicated bedside inoculation of media), an additive to liquid blood culture media, and indeed a self-contained culture medium that could be subcultured periodically during incubation to solid, agar-based media (12). Initially used at a concentration of 0.16%, later experiments suggested that concentrations higher than 0.05% could inhibit the growth of certain organisms (13). Liquoid essentially replaced saponin and trypsin as a blood culture anticoagulant over the next few decades, although the most effective final concentration and its use for the recovery of anaerobes remained an issue of some debate (12).

At about this same time, an interesting protocol for the recovery of streptococci was reported (14). This method (credited to Cecil without reference) called for the collection of 20 ml of blood in two tubes that were allowed to clot overnight in the icebox. The clots were then disrupted with a glass rod and placed in 50 ml of beef heart infusion broth and incubated at

37°C for a month. Subcultures were made every fifth day to blood agar and broth. The authors noted that the risk for contamination was high.

By the end of World War II, blood culture techniques would be fairly recognizable by clinical microbiologists today with a few exceptions. Blood samples were to be collected when patients exhibited temperature spikes, although the logistics of doing so would make timing a nightmare (15). Several (number not stated) specimens should be collected at hourly intervals to increase the likelihood of obtaining a positive result. Special consideration should be given to the choice of vein for collection. Drawing from a vein draining an infected area should be used whenever feasible. Blood collection from the femoral artery was believed to be better than venous blood for culture, although this too was disputed. If blood cultures proved negative, one could try sternal marrow as an alternative. It was recognized that pneumonia and meningitis were associated with bacteremia in about 50% of cases and duplicate bottles should be obtained in cases of subacute bacterial endocarditis, puerperal sepsis, and subacute focal infections, one bottle each being incubated aerobically and anaerobically. The latter was always to be included for patients with postsurgical infections using thioglycollate broth. Once collected, blood cultures should be examined once daily for 3 weeks. If *Brucella* spp., *Neisseria gonorrhoeae*, or *Neisseria meningitidis* infection was suspected, the atmosphere of the bottles should include 10% CO_2.

To avoid contamination, the following process was suggested. Blood should not be collected within 8 h of any intravenous injection. All electric fans should be turned off and windows and doors closed. The best vein for the infectious process at hand should be selected and a sterile towel placed under the collection site. Wash the site with soap or tincture of green soap in a circular motion. Remove the soap with a sterile gauze. Apply tincture of iodine or merthiolate over the area and cover with a sterile gauze until dry. Wash this off with 95% alcohol and allow to dry under a sterile gauze or towel. At bedside, withdraw about 15 ml of blood and inoculate a bottle containing 150 ml of dextrose beef infusion hormone broth with 5 ml of the total volume collected. Place another 5 ml in a citrated tube and mix to prevent clotting. In the laboratory, prepare two pour plates with 1 and 2 ml of citrated blood mixed with melted beef infusion agar cooled to 45°C and poured into sterile petri plates. Allow to harden, and label the volume of blood in each (these will be used to estimate the level of bacteremia). Place another 2 ml of blood into 25 ml of thioglycollate glucose broth for anaerobes. Prepare a Spray dish. (Note: a Spray dish is like a pineapple upside-down cake. It is a pour plate with the agar-blood mix in the top of the petri

dish, which is then covered by the bottom of the dish and inverted.) A mixture of sodium bicarbonate, sulfuric acid, and water is added to the bottom to generate CO_2 for cases of suspected *Brucella* spp., *N. gonorrhoeae*, or *N. meningitidis* infection. The plate is then sealed with paraffin and incubated. All cultures are incubated at 37°C for 24 h, examined, and an initial report generated based on Gram stains of the broth cultures which are subsequently subcultured to blood agar slates. This process is repeated every 2 to 3 days for 10 to 21 days until being reported as negative.

Scott's method simplified things a bit (16). It used 2-oz.-square, clear glass bottles with a screw-cap top and a rubber septum underneath. The aerobic bottle consisted of 15 ml of melted Trypticase soy agar dispensed into the bottle, which was turned on its side and cooled. Then, 10 ml of Trypticase soy broth was added to the bottle to create a modified Castaneda bottle (Fig. 4). Various iterations of this type of a system would be seen in later years, e.g., the Septi-Chek system from Becton Dickinson (Sparks, MD). The anaerobic bottle consisted of 25 ml of thioglycollate broth that had been autoclaved and stoppered to remove O_2. The aerobic/anaerobic bottles were inoculated with 5 and 2 ml of blood, respectively (the difference

Figure 4 The advent of the modern blood culture set of E. G. Scott (16) consisting of a thioglycollate anaerobic bottle and a broth/agar slant combination bottle for aerobes fashioned after the *Brucella* bottle designed by Castaneda (17). The bottle set on the left demonstrates no growth in either bottle, while the set on the right shows growth in both. Reproduced from reference 15, with permission.

in volume was not elucidated). *p*-Aminobenzoic acid (PABA) and penicillinase could be added if the patient was receiving sulfonamides or penicillin, respectively (first antimicrobial removal system?). The bottles were again incubated at 37°C and examined at 24 h and every 2 days thereafter. The agar side of the aerobic bottle could be "washed" with the blood/broth mixture if no growth was observed and the atmosphere of the bottle could be recharged by venting in the presence of 10% CO_2. Most isolates were recovered in 24 to 72 h unless antibiotics were on board. This method was also amenable for bedside inoculation.

If the blood sample clotted, serum was removed and the clot was placed in the barrel of a 10- to 20-ml syringe. The plunger was replaced, and the clot was forced through the tip of the syringe into a 100-ml bottle containing 50 ml of 0.12% beef infusion agar (semisolid).

Recognized contaminants of the day included diphtheroids (19% of the total) and skin cocci (*S. albus*, 24% of the total). Scott reported an overall contamination rate of 4.7% using his system, which included diphtheroids, *S. albus* (*S. epidermidis* and coagulase-negative staphylococci), *Escherichia coli*, and *Proteus* spp. One has to wonder about the latter two being designated contaminants.

Other standard blood culture media of the mid-20th century included hormone broth with 0.2% glucose, peptone colloid medium, tryptose phosphate broth, and Kracke's blood culture media designed to fix complement (15).

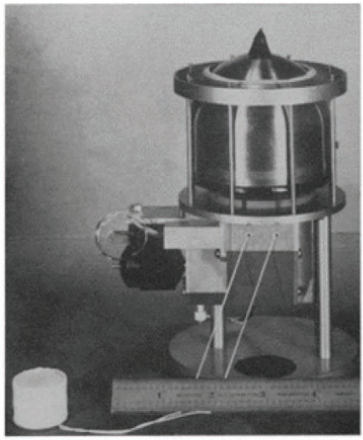
In all fairness, the first description of a biphasic liquid-solid medium interface was made by Castaneda in 1947 (17), and specifically designed for the recovery of *Brucella* species from blood. But, with time, it gained a more general appeal for the recovery of most organisms, including fungi and intracellular organisms causing bacteremia. Unfortunately, the popularity of biphasic blood culture bottles was offset by the complexity of producing them.

Advances in blood culture technology progressed at a glacier pace over the next decade, but two notable events paved the way for more significant developments down the road. The first of these seems like a non sequitur, but bear with me, because the connection will be made later. Levin et al., in a 1962 *Science* article, described a system developed by the National Aeronautics and Space Administration (NASA) by which microbial life in Martian soil could be detected using radiospirometry (18). The system was to be loaded on board a small probe called "Gulliver," which was intended to be delivered to the surface of Mars. Once there, the probe would launch two

cylindrical projectiles attached by 23 feet of wire onto the surface and reel the projectiles back into the probe, collecting soil samples in the cylinders in the process (Fig. 5). The contents would then be emptied into a microbial growth chamber containing a defined broth medium supplemented with glucose-^{14}C (uniformly labeled) and formate-^{14}C. If microorganisms were present in the sample and they utilized glucose or formate as a carbon source for growth, detectable levels of $^{14}CO_2$ would be generated and detected (after precipitation as barium carbonate) by a solid-state beta detector coated with barium hydroxide and situated directly above the growth chamber. Field tests on terra firma showed that the system worked with a wide variety of aerobic, microaerophilic, and anaerobic microorganisms. The system was also equipped with a nonlabeled CO_2-generating system that permitted flushing of the growth chamber prior to inoculation to eliminate any labeled gas that was generated during the long trip to Mars. This same approach would appear later as a microbial growth detection system specifically applied to the detection of microorganisms in blood cultures.

Similar to the Gulliver story, the second involved the development of a "rapid" blood culture method and modeled the way for future advancements years later. Winn et al. (19) described a novel membrane filtration blood culture technique that was more extensively evaluated later by Finegold et al. in 1969 (20). Although quite labor intensive, the method demonstrated the value of separating microorganisms from whole blood via dextran sedimentation of erythrocytes followed by differential capture of bacteria and yeasts on a filter and direct plating on solid media. The process allowed for removal of antimicrobial substances, promoted more rapid growth, provided discrete colonies for quantitation and rapid identification, and, hence, better discrimination between potential contaminants and significant isolates. It also allowed for the recognition of multiorganism bacteremia. Despite being more rapid than standard broth cultures (average time-to-detection 28 h versus 61 h for broth), the latter proved to be more sensitive for overall detection of microorganisms in blood.

In a hallmark proof-of-principle publication in 1969, Deland and Wagner (21) connected the dots between radiospirometric detection of bacteria on Mars and automated blood culture technology. Using a rudimentary closed-circuit system composed of a culture flask, circulating pump, and an analog ionization chamber connected via Tygon tubing to a readout meter and recorder, they were able to demonstrate active bacterial metabolism in liquid medium through the release of radiolabeled CO_2 (Fig. 6). The culture medium consisted of 20 ml of glucose-free thioglycollate broth supple-



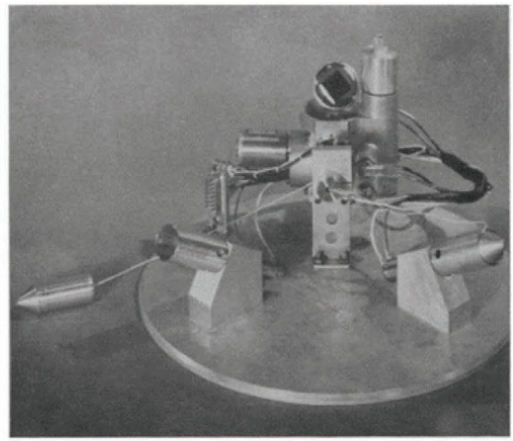


Fig. 6 (left). The first model of Gulliver, designed to probe for microbial life on Mars. Fig. 7 (right). The most recent model of Gulliver. This view shows the projectiles used to carry the soil-sample retrieval lines, the reel-in motor (at left), the broth chamber (at right and center), and the end of the solid detector, resting on the top of the culture chamber. The instrument shown has been used for field testing.

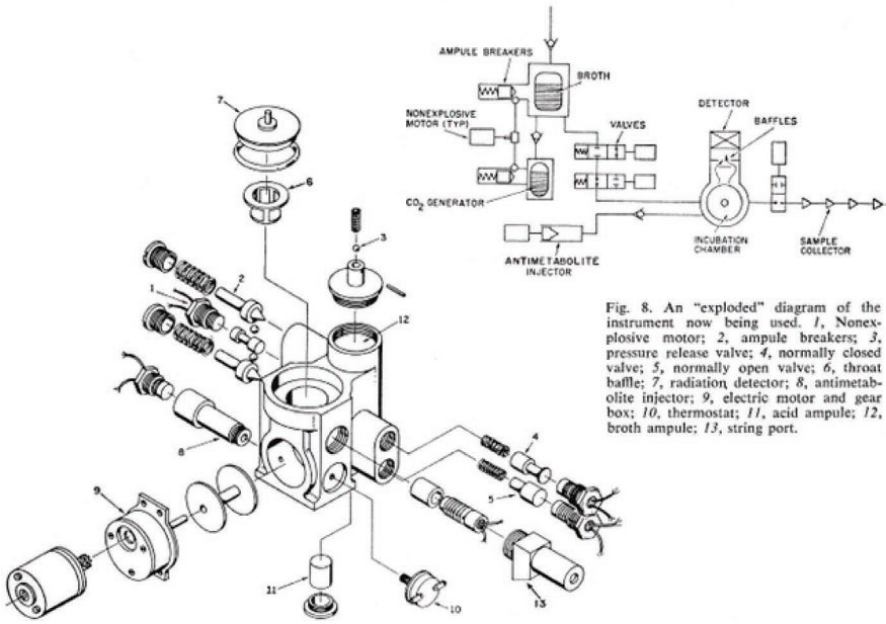


Fig. 8. An "exploded" diagram of the instrument now being used. *1*, Nonexplosive motor; *2*, ampule breakers; *3*, pressure release valve; *4*, normally closed valve; *5*, normally open valve; *6*, throat baffle; *7*, radiation detector; *8*, antimetabolite injector; *9*, electric motor and gear box; *10*, thermostat; *11*, acid ampule; *12*, broth ampule; *13*, string port.

Figure 5 Working models (above) and mechanical and schematic views of the Gulliver system, a radiospirometry system designed to collect soil samples on Mars, inoculate broth containing ^{14}C-labeled carbon substrates, and detect the evolution of $^{14}CO_2$ in an ionization chamber. Reproduced from reference 18, with permission.

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