

## OPPORTUNITIES AND RESPONSIBILITIES OF THE REFERENCE CENTER\*

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The title of this talk must seem forbidding to many of you. A reference center is a service organization which on the surface, and especially to the uninitiated younger scientists, connotes a museum with doddering curators. I hope today to counter that image and to impart a philosophy which has been successful in the hands of those with whom I have been fortunate enough to collaborate over the past 15 years, and which will be essential in the future if reference centers are to continue to be effective.

As you may guess, I shall draw heavily from my own experience with the World Reference Center for Arboviruses at the Yale School of Medicine. The same philosophy with principles and corollaries extends to a wide variety of similar programs which most of you either operate or interact with. I have in mind for instance the World Health Organization Centres for Research and Reference, the American Type Culture Collection, the Pan American Health Organization designated dengue reference center at the Walter Reed Army Institute of Research, the Research Reference Reagents Program of the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, the developing National Institutes of Health programs for cryopreservation of filarids such as *Wucheraria bancrofti*, the repository for *Aedes* mosquitoes at Notre Dame University, the Center for Disease Control regional center for arboviruses at Fort Collins, Colorado, the National Institute of Allergy and Infectious Diseases tick collection at the Rocky Mountain Laboratory, and the U.S. Department of Agriculture facilities in Ames, Denver, and Plum Island. In fact, each of you who maintains a cell culture, an animal, an insect, or a microorganism in your research program is in reality a reference center.

Let me sketch the background of the Arbovirus Reference Center at Yale, then I shall share with you my personal biases about the responsibilities and opportunities which a reference center affords.

Reference centers do not begin as reference centers. They begin as collections of scientists with a common research interest, and recognition that protoplasm in the form of type species must be conserved. They require a physical and financial resource sufficient to characterize and maintain type species, and a spirit to cooperate and share freely with others. I cannot emphasize too much that a reference center cannot succeed without cooperation and sharing.

The center at Yale was an outgrowth of The Rockefeller Foundation's program on arthropod-borne viruses. The Foundation in 1953 set up a world-wide network of laboratories<sup>1</sup> to study the distribution, epidemiology, and disease potential of viruses biologically transmitted by mosquitoes, ticks, and other biting arthropods. A base laboratory was also established in New York City, directed by the late Max Theiler. The New York City laboratory trained personnel in serologic identification techniques, many of which were actually developed there. It also produced reference reagents and received viruses for identification. A concept was adhered to in the Rockefeller Foundation field laboratories—no exotic viruses were to be introduced. This dictum insured that the agents sent to the reference center were isolated in the field and also originated there; the new viruses could not be laboratory contaminants.

At about the same time as The Rockefeller Foundation program started, other organizations including the U.S. Army, the U.S. Navy, the U.S. Public Health Service, and several universities and foreign governments embarked on similar field programs. In many cases, viruses were referred from these programs to the New York Laboratory for identification, and a serological classification of arboviruses was refined.

In 1965 the New York Laboratory was moved

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to the Yale School of Medicine under the direction of Wilbur Downs and was designated formally by the World Health Organization as the International Arbovirus Reference Centre.

The Yale laboratory today maintains a collection of over 400 distinct serotypes of arboviruses and other zoonotic viruses including the arenaviruses. Virtually all described arboviruses and many other zoonotic viruses are maintained except for those prohibited by the U.S. Department of Agriculture—Rift Valley fever, African swine fever, African horsesickness, exotic bluetongue types, and Nairobi sheep disease—and those zoonotic viruses so hazardous that they can be worked with only in maximum security facilities—Machupo, Lassa, Ebola, and Marburg.

I would like, now, to give you several examples of the opportunities presented by reference center material, each of which illustrates a principle or a significance factor which enriched the reference center experience.

The first principle is: *interaction among many scientific organizations is essential to exploit the full wealth of a reference center.* In October 1977 I received a phone call from Jack Schmidt of the Navy Research and Development Command. Jack said that one of their virologists at NAMRU-3 in Cairo, James Meegan, working with acute phase sera supplied by Imam Zaghloul Imam of the Egyptian Ministry of Health, had isolated a virus from fever cases from Sharquia and Qalyubia Governorates northeast of Cairo. Could Yale help identify the virus? We receive dozens of such requests each year but what made this different was the magnitude and seriousness of the problem. As we were to find out, about 200,000 persons were ill and by official count, 600 had died or were to die.<sup>2</sup> My notes of the phone conversation indicate that we discussed the differential diagnoses: dengue and yellow fever, unlikely in the absence of *Aedes aegypti* in lower Egypt; Lassa and Marburg viruses, unlikely to kill mice in the pattern observed; Bunyamwera and Semliki Forest viruses, do not usually kill persons; and Rift Valley fever, fits most of the observations, but what would it be doing in Egypt where it had never before occurred?

While waiting for Jim Meegan's arrival from Egypt, I tried to locate a Rift Valley fever antiserum. The virus and its antiserum are not readily available in the U.S. Fortunately, with tremendous foresight, Wilbur Downs in 1967 had contacted W. P. Allen, then with the U.S. Army at

Fort Detrick; Allen had supplied 40 ml of Rift Valley fever sheep antiserum which had passed safety tests and was received with U.S. Department of Agriculture permission. It had been virtually unused (but not forgotten) stored in the freezer for 10 years. When I asked Wil Downs why he requested it, he answered "I knew we would need it," and indeed we did.

Working in vertical laminar flow biosafety cabinets, we inoculated the virus into baby mice and BHK-21 cells. The complement-fixation test of Jim Meegan and Jordi Casals, using mouse antigen, was positive for Rift Valley fever almost simultaneously with the hemagglutination-inhibition test using mouse serum antigen and with electron microscopy by Owen Wood which showed bunyavirus particles in the BHK-21 cells. The diagnosis was transmitted simultaneously to the World Health Organization, Geneva; through the U.S. Navy to Egyptian authorities; and to the foreign quarantine authorities of the U.S. Department of Agriculture and the U.S. Public Health Service. The agriculture inspector was at Yale by 8 o'clock the next morning to supervise disinfection of the hoods in which the work was done. The Rift Valley fever strain was shipped to Plum Island and to Fort Detrick; the remaining materials at Yale were autoclaved. No fewer than seven organizations helped in the identification. Within 3 days the diagnosis was secure, but it could not have been made without a tremendous reference resource built on cooperation of many organizations and years of preparation.

The second principle is: *cooperation between veterinary and medical professions is essential to progress.* This is not an original concept, but one which we tend to forget. I shall continue the Rift Valley fever story. The Egyptian health authorities rapidly determined that huge numbers of cattle, sheep, and camels had aborted or died of the disease, and its spread to Egypt stimulated workers at Plum Island to test U.S. Army-produced vaccines in sheep and cattle both for altruistic reasons *vis à vis* world needs, and for U.S. readiness should the disease be introduced to our soil. We at Yale retained a vicarious interest in Rift Valley fever.

But opportunities arise under the strangest circumstances. Jerry Callis and Jerry Walker of the U.S.D.A. Plum Island Animal Disease Center phoned during late July last year. There had been an airline strike affecting J. F. Kennedy airport. Some Nile rats from the Sudan had come through

U.S.D.A. quarantine inspection there, but were delayed, then finally shipped on to Denver where they were to be used in rat control experiments. On arrival in Denver, some rats were dead and others were sick. I was told by Dr. Walker that an animal caretaker in Denver had also taken ill with fever and malaise. Could this be an importation of Rift Valley fever virus in the Nile rats? Two of the sick rats had recovered and their blood, along with some inactivated Rift Valley fever hemagglutinating antigen produced under auspices of the Army Research and Development Command, were available for testing. I agreed to do the tests.

This request again was similar to dozens we receive every year for reference service. The results are often negative and reside forever in our notebooks with a brief negative report to the organization which requested the test. In this case there was some urgency since an introduction of Rift Valley fever constitutes both a veterinary and a medical emergency.

I collected the specimens at the airport on Thursday afternoon and performed the hemagglutination-inhibition tests the next day. I included antigens to Wesselsbron, Germiston, chikungunya, and the phlebotomus fever group virus, Saint-Floris, since these agents could logically have been encountered in rats from Sudan and might make an animal caretaker sick. A totally unexpected result was observed. The phlebotomus fever grouping antibody, included to control the reactivity of the Saint-Floris antigen, inhibited the Rift Valley fever antigen. As so often happens the rodent sera were negative, but here was Rift Valley fever, thought by me and everyone else to be unique and unrelated, revealing its secrets. It was a member of the phlebotomus fever serogroup. By Monday we had the confirmatory results which have now been published.<sup>3</sup> The finding stimulated an entire new set of areas of research in vaccine development, epidemiology, genetics, and biochemistry, the results of which are not yet fully evident. The finding was only possible because of 1) cooperation between the veterinary and medical professions, 2) the availability of the resources of the reference center, and 3) the willingness to do tests without expectation of great rewards, a principle which is sometimes the most difficult to inculcate.

There is another important concept: *the Reference Center can and should be used regularly to certify the identity of infectious agents.* Every

microbiologist starts an experiment with an infectious agent. It may seem simplistic to state that the agent should be identified before the experiment and even during and after the experiment. This may be easy with parasites but is not so easy with viruses. We have discovered instances of mistaken identity or contamination in our own laboratory, and in a surprisingly large percentage of materials tested from other virus laboratories. This does not mean they are poor laboratories; on the contrary, the best laboratories request independent certification. Probably the best documented series originated in the program of the Research Resources Branch of the National Institute of Allergy and Infectious Diseases.<sup>4</sup> The Reference Center at Yale was commissioned to find out if the arboviruses used as starting seed preparations for reference reagents were correctly labeled and were pure, i.e., without contaminants. The wisdom of the NIAID was shown when we uncovered over 10% of the reagent sets with one or another complication. The tests involved: 1) neutralizing the virus with an immune serum from another isolate of the same virus to find a contaminating agent which would break through the immune barrier; 2) testing the immune reagent for contaminating homologous or heterologous viruses; 3) testing both the seed virus and the immune reagent from the seed virus for reaction with reference antigen and antibody of viruses known to be in the producer's laboratory; and 4) testing by complement-fixation the resulting immune reagent with a battery of 181 antigens of mouse pathogenic viruses. The results included such surprises as finding: a seed virus which was a mixture of two unrelated arboviruses; the inadvertent introduction of an extraneous arbovirus into the immunizing antigen; a contaminating homologous virus in the immune reagent; several contaminating murine viruses; and the mislabeling of bottles by the packager of the immune reagent.

Reference centers have helped investigators avert major errors of interpretation of experiments. The most common happening in our experience with arboviruses is contamination with an alphavirus. Semliki Forest virus made major headline news during 1980 when it was cloned using recombinant DNA technology following a presumed cross-contamination of Sindbis virus. Had the starting material been submitted to a reference center, the mix-up could have been recognized early. Drs. Casals and Buckley at Yale have detected eastern encephalitis in a putative

Sindbis preparation, Kunjin in a putative Murray Valley preparation, and Semliki Forest and chikungunya viruses on several occasions in supposedly normal *Aedes* cell lines. In each case the investigator who submitted their material to the reference center for testing was saved the embarrassment or time and effort entailed by a mix-up, and none of these cases have become headline news.

*Reference center findings can have sudden and unexpected repercussions on world veterinary trade.* Australia exports three billion dollars' worth of livestock and livestock products annually.<sup>5</sup> During the summer of 1977 Christina Frazier, working with me as a post-doctoral fellow, called me over to see the results of a complement-fixation test. I had suggested that she try to identify some unknown viruses from Australia. I had second thoughts about saddling her with rather routine identification of viruses, but she was enthusiastic and insisted that she would help out in addition to her regular research program. The complement-fixation test showed that specimen CSIRO-19 was bluetongue virus.<sup>6</sup> After working the week-end to repeat the finding, we cabled Ralph Doherty of the WHO Regional Centre for Arbovirus Reference in Brisbane and Toby St. George of the Commonwealth Scientific and Industrial Organization Veterinary Laboratory. The significance of this is awesome, when one realizes that bluetongue was not known in Australia and the inevitable result would be embargo of the three billion dollar livestock export trade by Australia's friends.

Now I wish there had been some better way to let our Australian colleagues know because, as expected, the impact was sudden and terrible. We could not just whisper the news though, because we also had a sacred responsibility to APHIS, the U.S.D.A. quarantine authority, to let them know we had been studying in New Haven, albeit unwittingly, a class 5 exotic animal pathogen.

Predictably, the cost to Australia was high—about \$3,000,000,000.<sup>5</sup> Ironically, CSIRO-19 virus when it was tested for its ability to cause disease in sheep and cattle, was nearly benign.<sup>7</sup> Yet infection and disease are not differentiated in the reporting requirements of OIE, which mandates that the 16 diseases on their List A be reported immediately by member countries for international notification.<sup>5</sup>

In the case of Australia, the identification of bluetongue virus by the Yale Reference Center led

to: 1) complete prohibition on the import of live ruminants by U.K. and New Zealand; 2) a ban on imports of meat by U.S.S.R. in spite of the fact that large shipments were already in Soviet waters in 1977 when the announcement was made; 3) partial embargoes on shipment of live sheep to the Middle East which imported nearly 5,000,000 head from Australia in 1978; and 4) a temporary ban by the People's Republic of China on the import of wool and hides.<sup>5</sup>

The Australian authorities, I am happy to report, were understanding of the reference center's responsibilities, the loss of \$3,000,000,000 notwithstanding. In fact since 1978 they have made an annual grant to support the Yale Reference Center. In addition, the Australian scientific community has responded to its plight by making major scientific advances in bluetongue research since the identification of bluetongue virus in Australia in 1977.

Let me now relate another Australian experience which illustrates that *the reference center findings can foretell the future.* In 1956, Dr. S. G. Anderson of the Walter and Eliza Hall Institute of Medical Research, Melbourne, collected 16 pairs of acute and convalescent sera from patients suffering from epidemic polyarthritis and rash. The patients became ill during an epidemic of one to two thousand cases which occurred in Mildura in the Murray Valley.<sup>8</sup> Not only were these people quite sick with debilitating and painful arthritis, but also the disease occurred during the vacation season and the area suffered financially. Dr. Anderson sent the sera to Max Theiler and independently to the late Kenneth Smithburn in South Africa. Dr. Theiler asked me to examine the sera to see if antibody to chikungunya virus were present. We both knew that chikungunya virus caused in Africa a disease somewhat like the Australian epidemic polyarthritis and rash. Theiler, in his wisdom, failed to tell me that Smithburn had already tested the sera by neutralization test and had found them negative with chikungunya virus.

Influenced by Jordi Casals and Loring Whitman, I designed the tests differently from Ken Smithburn in two ways—I used the hemagglutination-inhibition test which cross-reacts broadly within the alphavirus genus, and I used two alphaviruses from the reference center collection, Bebaru and Getah from Malaya, viruses which I had previously studied there in 1957 when I was a member of the U.S. Army Medical Research Unit. The test results showed serologic rises in

titer in six of the pairs to Bebaru and Getah viruses. We stated: "The fact that six of 16 patients developed antibodies to group A arthropod-borne viruses during attacks of polyarthritis and rash in Mildura in 1956 is presumptive evidence that the epidemic was caused by an arthropod-borne virus related to those used in serological testing. The pattern of HI response, however, is not one that would be expected from any given single virus among those used in testing. Accordingly, it seems probable that the virus isolated will be found to differ from the existing identified members of group A."<sup>8</sup>

In 1963, the isolation of Ross River virus from *Aedes vigilax* mosquitoes was reported by Doherty et al.,<sup>9</sup> who also showed the Ross River antigen to be excellent for diagnosing epidemic polyarthritis and rash. Ralph Doherty suggested that Ross River virus was itself the cause of the diseases. Only last year,<sup>10</sup> 20 years after the original prophecy did Rosen, Tesh, Gubler, and investigators from New Zealand and Australia isolate Ross River virus strains in large numbers from blood of patients in Fiji, Samoa, and other Southwest Pacific islands. The prophecy was fulfilled.

*A reference center must receive material from as wide a variety of sources as possible.* Imagine if you will, a virus isolated in 1964 by Jack Schmidt from *Mansonia uniformis* mosquitoes near Malakal, Sudan;<sup>11</sup> a virus isolated in 1956 by Drs. Boulger and Porterfield from *Eidolon helvum* fruit bats in Lagos, Nigeria;<sup>12</sup> a virus isolated in 1968 by Graham Kemp from *Crocidura* spp. shrews originating near Mokola Circle in Ibadan, Nigeria,<sup>13</sup> and subsequently from human cerebrospinal fluid;<sup>14</sup> and a virus isolated in 1968 by Vernon Lee from *Culicoides* midges captured from cattle at the University of Ibadan farm in Nigeria.<sup>15</sup> At the same time imagine that Fred Murphy of CDC, Atlanta, and I are jointly studying systematically, and I confess in a rather unimaginative fashion, the known rhabdoviruses of animals including rabies virus. Fred examined the ultrastructure and I the serological reactions. We were both independently doing similar procedures with unidentified viruses received for reference study.

One day the pieces of a puzzle suddenly fell together. Fred telephoned to say that Lagos bat virus was a rhabdovirus by electron microscopy. He said, "It looks like rabies; could a mistake have been made?" I said, "No, because I have just shown by complement-fixation test that Lagos bat

virus is related to, but different from a virus from a shrew"—Kemp's virus which was later to be named 'Mokola'—"and rabies does not have any serorelatives." I had also just found that Mokola virus was related by complement-fixation test to the virus from Sudanese mosquitoes (subsequently named 'Obodhiang') and how could rabies virus come from mosquitoes?

The whole set of relationships was so beset with coincidences and so anti-dogma that I shared the findings only with Wil Downs and a few other close friends (and I am sure Fred Murphy also was silent) until later when we established that these viruses were indeed related to rabies both serologically and ultrastructurally.<sup>16</sup> Dorothy Moore subsequently placed the *Culicoides* isolate, kotonkan, in the rabies serogroup,<sup>15</sup> and Tignor et al. identified another rabies-related virus, Duvénhage, which was isolated from a rabid South African man bitten on the lip by a bat.<sup>17, 18</sup>

It was only through efforts to maintain a wide-ranging network of collaborators that it was possible to bring together this unlikely meld of viruses into what now appears to be an evolutionarily sound scheme, the rabies serogroup, recently dignified by a genus name, *Lyssavirus*.

A corollary to "*A reference center must receive material from as wide a variety of sources as possible*" is "*never neglect uninteresting material.*" The agent with the lowest priority for characterization may be the missing piece in the taxonomic puzzle. The finding of the rabies serogroup stimulated research on four continents and has led to the once revolutionary, but now accepted, concept of antigenic diversity of rabies virus and the hypothesis that perhaps different vaccines are needed for different parts of the world.<sup>19</sup>

A principle which cannot be overemphasized is *the reference center collection must be ready as an invaluable resource when new technology appears.* Possibly the most important opportunity offered by a reference center, especially world reference center, is application of new technology to a large and varied collection.

It is little known that Max Theiler had grouped the orbiviruses more than 20 years ago. He was fascinated with the effect of bile salts on viral infectivity,<sup>20</sup> and for years tested each new virus that was referred to the arbovirus center. He thought arboviruses should be sensitive to bile salts and could not understand a virus set which was obviously arthropod-borne but showed only slight sensitivity. The exceptional viruses were

recorded one by one in a separate section of his notebook. One of these was Colorado tick fever virus.

I do not recall the exact sequence of events, but I know Fred Murphy and Ernie Borden at CDC, Atlanta, were busily applying thin-section electron microscopy during the 1960s; maybe it was not strictly speaking a new technology, but certainly it was not widely applied before this to arboviruses. They showed that Colorado tick fever virus had the characteristic morphology we now attribute to orbiviruses—cytoplasmic, icosahedral particles with accompanying matrix and tubular forms. The special section in Max Theiler's notebook suddenly assumed significance. Could these bile-resistant viruses also be like Colorado tick fever virus?

Max Theiler carefully located each of these agents, gave them to me for serologic comparison, and I in turn shipped each to Atlanta for electron microscopy. Every one was an orbivirus. Within 6 months the entire genus was characterized and named. Although Max Theiler steadfastly refused recognition through authorship on the publications,<sup>21,22</sup> it was his systematic bile sensitivity testing of viruses from the collection that established the basis for the genus. The collection had been ready and waiting when the electron microscopy technology applied by Fred Murphy was ripe.

The arbovirus collection continues to be used effectively, a recent example being the extensive genetic and molecular studies of the family Bunyaviridae being done at the University of Alabama and at Fort Detrick.<sup>23,24</sup>

*Some future responsibilities and opportunities.* We are going through a period of rapid technological advance. The reference centers of the world must keep up. The storage and classification of type species, whether they be insects, viruses, protozoa, or other parasites, will remain as a basic function. There are two major technological advances which promise to generate enormous numbers of new collections: recombinant DNA and monoclonal antibodies. Certain products of these technologies are commercially attractive, as reflected by the rapid advance in the price of Genentech stock during 1980. You can bet these commercial products will be preserved well by industry as long as profits are to be had. There will be many other products useful to tropical medicine—cloned viral and parasitic products, and hybridomas which produce monoclonal

antibodies. Many of these are of primarily scientific rather than commercial value. It is time now to refine and to implement inexpensive methods of preservation and to prepare repositories.

Existing type culture collections and reference centers are technically equipped to maintain these new collections. There is no question about the need. The challenge is firstly in the willingness of investigators to share freely their new-found products and secondly in the ability of reference centers to handle what promises to be massive numbers of new acquisitions. I believe we are up to the challenge.

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