SYMPOSIUM ON VIRAL AND RICKETTSIAL DISEASES[1]

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[1] Presented at the Golden Jubilee Meeting of the Society of American Bacteriologists at Baltimore, May 15, 1950.

Joseph E. Smadel was the convenor and has served as editor of these papers.

PART I

1. INTRODUCTION

JOSEPH E. SMADEL

The subjects of the papers of the Symposium on Viral and Rickettsial Diseases held at the Golden Jubilee meeting of the Society of American Bacteriologists were selected to cover a wide range of interests. Certain of the topics are concerned with such basic aspects of the science of virology as purification and reproduction. Other subjects, of more immediate importance to general medicine, have to do with diagnosis, prevention and control of viral and rickettsial diseases. Two of the papers integrate the recently acquired knowledge of two general groups of viral agents: (a) the dengue viruses and their distant cousins, and (b) the poliomyelitis virus together with several families of agents which, though unrelated directly to this virus, have been associated with certain of the clinical findings sometimes manifested by infection with the agent of poliomyelitis. Finally, two of the papers describe recent developments in the field of rickettsial diseases.

The nature of viruses remains an enigma but curiosity continually stimulates investigators to attempt to understand these most minute of living things. Viruses and rickettsiae will grow only in living cells, hence, all initial preparations of these agents are heavily contaminated with normal cellular components. Indeed, it would be more accurate to say that preparations of host cell material are contaminated with virus. The need for purified preparations of viruses for studies on the physical, chemical, biological and immunological nature of these substances is apparent. Hence, it is not surprising that many investigators have devoted much time and effort in attempting to attain these. Dr. Warren discusses the results which have been obtained in the purification of the animal viruses, in general a more recalcitrant group for such studies than the plant viruses. Furthermore, he devotes considerable time to the newer methods which may be useful in eliminating normal components from preparations of the small, mammalian viruses, a group which has been particularly difficult to purify.

Another fundamental problem in this field concerns the methods by which viruses reproduce. Virologists with the judgment to employ the simplest of the virus-host cell systems, i.e., bacteriophage and bacterial cell, have contributed most to our knowledge in this field. Dr. Evans, who reviews his own work and that of others, gives us considerable insight into the most private aspects of the pilfering lives of the phages. However, he is careful to point out that his observations are on the habits of certain phages and may or may not be applicable to other viruses and rickettsiae.

The general control of viral diseases continues to be one of the most important problems in medicine. Prevention of smallpox by Jennerian prophylaxis is more than 150 years old and Walter Reed's fundamental observations on the prevention of yellow fever by control of the mosquito vector were made 50 years ago. It is to be regretted that the past half century has provided no similarly satis-

factory methods for the control of any other virus disease. These failures are not from want of scientific effort nor from the lack of new ideas as Dr. Horsfall points out in his discussion of this subject.

For the past quarter century dengue fever has not been an important disease in the United States. Nevertheless, in the early 1920's an epidemic in our southern states affected well over a million people; such an outbreak might reappear at any time. Dengue fever continues to be of concern to many military and civilian populations in tropical and sub-tropical areas. Extensive investigations of this disease were undertaken during World War II, and the contributions of Dr. Sabin's group deserve a place in this symposium: they add to our knowledge of the viral agent; they contribute to our understanding of the duration of immunity to closely related but different infecting agents; and finally, they increase our appreciation of the modifications of the typical disease picture which may result from infection in a partially resistant host.

Although the monkey was shown early in this century to be a suitable experimental animal for work with the virus of poliomyelitis, relatively few laboratories actively investigated this agent until the past decade. The unprecedented lay interest in this disease in recent years has provided investigators with facilities for extensive studies on the virus of poliomyelitis and related agents. Dr. Melnick discusses certain of the newer information on the various strains of poliomyelitis virus. In addition, he summarizes the knowledge of two groups of viruses, i.e., encephalomyocarditis and Coxsackie, which were discovered during the course of work on poliomyelitis and which, following the initial period of confusion, have added to our understanding of infantile paralysis as well as diseases of the central nervous system.

Information on the rickettsial diseases was relatively slow in being acquired. Nevertheless, because of various factors, it is probable that more knowledge was accumulated in the past 15 years on these maladies than was gathered during the past half century on the virus diseases. It is true that most of these advances in rickettsiology are concerned directly with medicine, for example, diagnostic methods, immunization procedures, control of vector agents, and finally, therapy of patients. Abstract information on the nature of the rickettsial agents may be somewhat less voluminous than that of the viruses. However, rickettsial preparations of a degree of purity as great as any yet attained with the viruses have been available for a number of years (9). Furthermore, the rickettsiae have been shown to have at least one type of independent metabolic activity (5), the capacity to oxidize glutamic acid; it still remains to be demonstrated conclusively that any virus possesses an intrinsic mechanism for metabolizing any substance *in vitro*. A symposium on rickettsial diseases (1) held in 1946 brought together the main contributions in this field which had been made during the war years. The three important developments in rickettsiology since that symposium are (a) the discovery of a new rickettsial disease of man, rickettsialpox, (b) the finding of a relatively high incidence of Q fever in certain parts of the United States and the development of a considerable understanding of the epidemiology of this disease, and (c) the discovery of antibiotic agents which are highly efficacious

in the treatment of the rickettsial diseases. These new developments are discussed by Dr. Huebner and Dr. Lennette.

In the midst of this scientific banquet I have chosen to discuss the most unappetizing aspect of the diagnosis of viral and rickettsial diseases, i.e., the organization of a diagnostic service. Although the diagnostic procedures themselves are at a relatively adolescent stage of development, they are ready to stand on their own without the guardian care of their scientific parents or the protective shielding of the research laboratory. Investigators in this field should welcome the departure of the adolescent and turn their attention to the underdeveloped infant methods for the early diagnosis of these diseases.

2. LABORATORY DIAGNOSIS OF VIRAL AND RICKETTSIAL DISEASES, A REAPPRAISAL AFTER TEN YEARS

JOSEPH E. SMADEL

The papers of the other participants in this Symposium are truly scientific reports. Mine on "Laboratory Diagnosis" is really only a news letter. I shall talk mainly about the new entity, the "Virus Diagnostic Laboratory," which was conceived and developed during the past decade. Such laboratories provide an intermediate step between those devoted to pure research and those concerned with routine diagnostic procedures. However, we have already progressed beyond the virus diagnostic laboratory to the stage where any laboratory which is capable of performing ordinary serological procedures can now employ certain of the techniques for the diagnosis of viral and rickettsial diseases. Perhaps you were startled by the use of the word "progressed" in this connection. I assure you that it was carefully chosen, since it is my conviction that the real value of viral and rickettsial diagnostic procedures can only be realized when they are readily available to the physician and his patient. We shall come back to this subject later but let us now take up the problem of the virus diagnostic laboratory.

In the decade prior to World War II, basic information on the viral and rickettsial agents was acquired in the research laboratories and most of the principles were established on which the present diagnostic tests are based. Despite these significant contributions, the research laboratories of that period were interested in narrow problems and their efforts contributed little to the direct diagnosis of human disease and the ultimate care of patients. In 1939 and 1940 a clinical investigator could have obtained almost all of the viral and rickettsial diagnostic tests which are now available to him. However, it would have been difficult to get such diagnostic data since each research laboratory specialized in one or two procedures which had been developed under its aegis. Furthermore, while such laboratories occasionally condescended to do routine clinical diagnostic tests, it was always obvious that these were undertaken at the convenience of the investigator and as a personal favor to the clinician.

Impetus to the establishment of the virus diagnostic laboratory, as we now

know it, was supplied by the Army which laid plans for such an organization in 1940. The first of these was established at the Army Medical Department Research and Graduate School in 1941 under the direction of the late Colonel Harry Plotz. In this laboratory the various diagnostic procedures for the viral and rickettsial diseases were, for the first time, brought under one roof and capable investigators were given the task of applying them. This laboratory apparently fulfilled the Army's expectations since it was permitted to grow, and to develop by fission a series of daughter laboratories which were scattered over the world wherever our armies went. Certain of these military laboratories were closed during the period of demobilization, but even in the present era of uncertain peace, the Army maintains a number of these units.

As a result of developments in the field of civilian medicine in the past few years, the Army no longer has a monopoly on virus diagnostic laboratories. This is as it should be. There is no doubt that the practice of medicine in the United States has been improved as a result of the virus diagnostic laboratories which have been established by the State of California, by Philadelphia and the State of Pennsylvania, by the City and the State of New York, and by the Communicable Disease Center of the Public Health Service. Other laboratories of a similar nature are in the process of development and will assume their responsibilities to the people of their respective areas.

These virus diagnostic laboratories, of which I have been speaking, are generally devoted to research as well as to clinical diagnosis. This welding of two diverse interests results in a healthy symbiotic existence. Many of the problems encountered in such laboratories are beyond the capabilities of routine laboratories. Hence, their solution demands the equipment and personnel which characterize good research organizations. The presence of investigators in these laboratories permits us to expect a continuing improvement in the diagnostic methods as well as advances in the general field of infectious diseases.

The central virus diagnostic laboratory with its research and routine is now an established institution. At this point I wish to discuss at greater length the newest development in the field, that is, the ordinary serological diagnostic laboratory which has begun to perform certain of the more standardized virus diagnostic procedures.[2] Here again the Army has provided the lead. During the past five years the School has turned over to medical laboratories in the Army the responsibility for performing serological diagnostic tests for the influenzas, for the psittacosis-lymphogranuloma venereum group of infections, for epidemic and murine typhus, and for Q fever. The Army Medical School provides these laboratories with standardized antigens and antisera, and, from time to time, supervision and advice in the use of these materials. The results have been most satisfactory.

The application of this development to civil medicine is dependent upon

[2] It is not the present purpose to discuss the various technical methods employed in this field. These were given in detail by various authorities in "Diagnostic Procedures for Virus and Rickettsial Diseases," published in 1948 by the American Public Health Association, and are discussed in current textbooks of virology.

the availability to hospital laboratories of the necessary diagnostic antigens and control materials. Within the past few years, the majority of these materials have become available through the interest and efforts of the commercial biological houses. Problems still exist in the use of these commercial materials but answers will be obtained, and a more widely disseminated use of the serological procedures for the diagnosis of viral and rickettsial diseases is foreseen.

One may ask whether the wider application of the serological diagnostic procedures in the diseases under discussion will eliminate the need for the central virus diagnostic laboratory. It is my opinion that this will only increase the demand for the services of the central laboratory which is capable of performing the entire gamut of diagnostic techniques and of providing advice and consultation. There still remain many problems, particularly those associated with the isolation of infectious agents and the performance of the more difficult procedures, which are beyond the capabilities of the ordinary serological laboratory. One must also realize that studies limited to the use of the serological procedures with available materials will add little to the general knowledge of infectious diseases. If the work is restricted to this aspect alone, no new agents will be discovered and few ideas in the control of infectious diseases will germinate. Therefore, the hospital laboratory must have available for reference a central laboratory which will supplement its own efforts.

Should we be satisfied with the recent progress and the developments which may be expected from a continuation of the present lines of approach? Of course, the answer is no. For practical purposes, all of the present methods provide the diagnosis too late in the course of the individual patient's disease for the information to be of value to the physician responsible for his care. The reasons are simple. Our serological procedures are based upon the detection of specific antibodies. Therefore, the patient is usually well along in convalescence, or has died, before the diagnosis can be established. Our isolation procedures generally take even longer than the serological ones because in almost every instance it is necessary to isolate the agent in some experimental animal host and then to establish its identity. Therefore, the need is for an entirely new line of approach which will provide a specific diagnosis at the time the physician first visits the acutely ill patient.

This deficiency in diagnosis is dramatically illustrated in the rickettsial diseases and in the pneumonia produced by psittacosis virus. Excellent therapeutic agents are now available which induce prompt recovery of patients with these infections (23, 24). In our experience in the treatment of scrub typhus (27), we found that the results of a therapeutic trial provided the most rapid means for obtaining presumptive confirmation of the clinical diagnosis. The available laboratory procedures were useful only in establishing a retrospective diagnosis for a cured patient. There are indications that the research laboratories may be on the verge of developing procedures which will satisfy the demand of the physician for early diagnosis.

Procedures for the very early diagnosis of rickettsial diseases were described in 1942 by León in Mexico (17) and Smorodintsev and his associates in Russia

(28). Both worked with patients who were in the first week of their infection with epidemic typhus. León was able to demonstrate a specific antigen in the urine and Smorodintsev in the blood of such persons. The methods employed by both investigators are difficult and probably are not suitable for use in the ordinary laboratory. However, both techniques have pointed the way for further developments. O'Connor of the Commonwealth Serum Laboratories in Australia has contributed recently to the early diagnosis of scrub typhus (19). His technique, which was capable of detecting minute amounts of antigen in the urine, was based on the work of his colleague Keogh, who in 1947 (13) showed that the adsorption on erythrocytes of certain bacterial antigens rendered these cells agglutinable by specific bacterial antibodies, and furthermore, that the prior addition of homologous antigen to the antisera inhibited the agglutination reaction.

There is still much to be done before procedures for the early diagnosis of viral and rickettsial diseases become usable tools for the physician. Nevertheless, the present information indicates the possible value of certain of the newer methods and warrants extensive investigation of these possibilities.

3. PROGRESS IN THE PURIFICATION OF VIRUSES OF ANIMALS

JOEL WARREN

Fifteen years have passed since Stanley reported that a nucleoprotein isolated from diseased Turkish tobacco plants possessed all the properties of tobacco mosaic virus and, more remarkably, existed in a crystalline state (29). The exciting implications of this observation and its profound reverberations in all spheres of biological research are still vivid in our recollections. The isolation of animal viruses of comparable purity, perhaps as crystals, was a prospect which appeared attainable within a short time. Within the next few years several more crystalline plant viruses were isolated; and a dozen or so enzymes, including catalase, papain, lysozyme and ribonuclease, were also crystallized.

The point of view became well entrenched that when the "pure" animal virus was obtained it too would have to be crystalline and a single, definable chemical substance. Lively debate (much of which, unfortunately, is unpublished) raged over such matters as the incompatibility between "living" and "crystalline" states or whether a pure nucleoprotein necessarily had to be a crystal. But these oratorical storms of the late 1930's have subsided and we are no longer lulled by the comforting thought that crystallinity is synonymous with purity. It has been replaced by the more pragmatic attitude of let us wait and see what each animal virus is like when there no longer exists reasonable doubt as to its purity.

Although the isolation of an unequivocally pure animal virus has not been accomplished and this objective appears more remote than it did fifteen years ago, considerable progress has been made towards this goal.

No longer do most workers regard animal viruses as closely akin to plant

viruses and equally amenable to procedures employed in plant virus isolation. Furthermore, there is considerable individual variation between the physical and chemical properties of different mammalian viruses. It is not heretical to state that when the basic mechanisms of multiplication in animal viruses are uncovered, even they will prove to vary from one virus to another.

This paper traces the more significant landmarks in the natural history of purification which determine our present concepts. Several excellent and more comprehensive reviews by other workers have recently appeared, notably those of Burnet (6), Smadel (25), Pirie (21), Beard (3), Lauffer (15) and others. We shall limit our discussion to the question of what trustworthy data have been accumulated from each reported "purification." Finally, we shall briefly mention the recently described protamine precipitation method and its application to the purification of some of the smaller viruses.

In 1942, Smadel stated: "The recent development of knowledge (of animal viruses) represents the flowering of a field carefully cultivated for half a century and sporadically tended for the preceding hundred years" (25). Specifically, the major investigations in the isolation of mammalian viruses seem to have had their most productive period shortly after the crystallization of tobacco mosaic virus. Curiously enough, the technique used for the separation of this plant virus from host tissues (precipitation by electrolytes or organic solvents, isoelectric precipitation, and adsorption on surface active substances) has had comparatively little application in mammalian viruses. In retrospect, the accelerated attempts at animal virus concentration seem more dependent upon the improvement in methods of centrifugation than any other one factor. The air turbines of Beams, Pickels, Wyckoff and others that came into widespread use around 1936 made possible a further continuation of the older observation of MacCallum and Oppenheimer in 1922 (18) and Ledingham in 1931 (16) that viruses could be separated from infected tissue suspensions by centrifugal force. Incorporating the optical methods developed by the Uppsala School into the ultracentrifuge made it possible for these beautifully constructed machines not only to concentrate the virus but to provide quantitative data on its physical properties as well.

The purpose of table 1 is to summarize briefly the chronological order of some recent attempts to isolate the animal viruses and establish their state of relative purity. You will note that most of the agents first studied are among the largest and those which can be isolated from infected cells in high concentrations; what Beard calls "the easy ones."

The greatest insight into the properties of mammalian viruses was obtained from the agent of vaccinia. Not only does vaccinia virus afford a particle of large size but it has been established that under the proper conditions a single elementary body is probably sufficient to induce disease (20). In the last years of the 1930's, highly purified vaccinia virus was obtained in considerable quantity and analyzed by most of the chemical and immunological techniques then available (25). The complexity of this agent was soon apparent. The finding of neutral fat, apparently an integral part of the infectious entity (11), was of

considerable interest for lipoids were not found in those plant viruses thus far studied.

Vaccinia virus was shown to have a high protein content, approximately 90 per cent, and the nucleic acid present was identified as a desoxypentose type (25). A second important concept emerged when vaccinia virus was found to contain small amounts of copper, flavin-adenine-dinucleotide, and biotin. This animal virus also possessed many of the constituents found in the host cell and was perhaps independently capable of some primitive energy exchanges.

The demonstration of any enzymic activity of the purified virus to serve as experimental proof of this thesis ran into a difficulty which still has not been surmounted. While certain enzymes could be detected in preparations of the highest attainable purity, it is impossible to state whether these reside within

TABLE 1

The chronology of purification of animal viruses

VIRUS	APPROXIMATE SIZE OF PARTICLE, Mμ	DATE "PURIFIED"
Vaccinia	210 x 260	1932
Rabbit papilloma	45	1939
Influenza A	115	1943
Influenza B	123	1943
Influenza, swine	117	1943
Eastern equine encephalomyelitis	25–40	1943
Murine encephalomyelitis	10–15	1943
Columbia-SK*	10–15	1944
Poliomyelitis (Lansing)	10–15	1946
Newcastle disease	40 x 500	1947
Mumps	200–260	1948
Japanese encephalitis	18–22	1949
Encephalomyocarditis*	10–15	1949

* These are different strains of the same virus.

the virus particle or are simply adsorbed on its surface from the host protoplasm (12).

The investigations of vaccinia yielded two more contributions to our basic knowledge of animal viruses: complexity of physical structure and complexity of antigenic composition.

As regards structure, it is perhaps fortunate that the application of the ultracentrifuge in virology preceded that of the electron microscope. Otherwise, it is not unlikely that the literature would be filled with a mass of photographs of those bizarre objects found in crude suspensions whose claim to the designation of "virus" reside only in the infectivity of the material. Thus, not only is their purity in doubt, but their antecedents are dubious and it would have required considerable labor to establish their legitimacy. Many of us have seen electron micrographs of vaccinia elementary bodies with their strikingly symmetrical

cuboidal structure and internal condensation of material. It is not necessary to dwell on a comparison of this structurally organized agent as contrasted, for example, with the crystals of tobacco mosaic or tomato bushy stunt virus.

Multiple antigens in an animal virus had long been known (8) but in vaccinia at least four distinct immunological components were demonstrated by means of serological tests and still other antigens were detected by *in vivo* procedures (26). In most plant viruses (15) and in rabbit papilloma virus there appears to be only a single antigenic constituent responsible for their immunological behavior.

At this point the school of thought which regarded animal viruses as some sort of macro-molecule (30) was confronted with the necessity of either discarding this thesis in the case of vaccinia or hypothesizing the "moleculae gigantibus" of protoplasm. Parenthetically, it may be noted that judging from current literature this point of view is rather decadent.

Investigations of purified rabbit papilloma virus by Wyckoff (38), and later by others (32), provided two fundamental pieces of data. This animal virus consisted largely of nucleoprotein and it behaved as a homogeneous molecular species when examined in the analytical centrifuge and in the electrophoresis and diffusion cells. Such observations were in accord with the properties of the plant viruses.

Continuing chronologically to influenza virus, two events contributed largely to its purification. In 1941 Hirst, and McClelland and Hare discovered the phenomenon of erythrocytic adsorption of this virus. Secondly, the mobilization of our troops in 1940–1942 was accompanied by the initiation of a program for the large scale production of influenza vaccines. Adsorption on and elution from chicken red blood cells supplemented by ultracentrifugation produced concentrates of sufficient purity to furnish new knowledge of the animal viruses.

First, examination in the ultracentrifuge and electron microscope revealed that influenza virus, unlike papilloma virus, exists as polydisperse particles with a distribution of sizes about a mode. Not only do particle sizes differ but their shape is also variable in infectious material (34). It has been suggested that the size diversity may be an artifact caused by the purification procedure and that different properties of the virus, e.g., hemagglutination, infectivity, or complement-fixation, are concentrated only in particles of a specific size (10), but proof or disproof of these possibilities must await further study.

Second, in influenza, an animal virus was found for the first time in which the carbohydrate content (approximately 12 per cent of the total dry weight) apparently exceeded that which could be associated with the amount of nucleic acid present (31, 14). The nature of this material has not been adequately studied. A specific characterization of this carbohydrate should be accompanied by a parallel analysis of the carbohydrates present in the chick allantoic fluid in which influenza virus is commonly cultivated.

Lastly, the property of an organized complex framework, first noted in vaccinia, was also revealed in electron micrographs of the influenza virus, within

which particles also are seen evidences of an internal structure. More recently, this heterogeneity of form among the animal viruses has appeared in the photographs of concentrated Newcastle (2) and mumps (37) viruses.

An observation which seems to narrow the gap between plant and animal viruses was forthcoming from the chemical analysis in 1943 (33) of the virus of equine encephalomyelitis. All of the plant viruses isolated have been found to contain ribonucleic acid and, up until 1943, all the animal viruses studied contained desoxyribonucleic acid. The equine virus, however, was found to contain ribonucleic acid in common with the plant viruses. It seems possible that some of this may have been a contaminant from the chick embryo.

We must digress for a moment here to the problem of contamination of purified virus preparations by materials derived from the host tissue, and in particular, by that group of heterogeneous substances now loosely designated as "normal tissue components." Granules have been reported from various normal mammalian tissues, allantoic fluid and milk, whose reported sizes of 20 to 200 mμ bracket those of the animal viruses. The chemical and physical properties of the large bodies are also closely akin to viruses and they contain ribonucleic acid. If these merely represent fragments of larger broken structures (7), then their homogeneity of size and form and heterogeneity of composition are truly remarkable. It seems more likely that some normal components, such as those of brain and allantoic fluid, are entities in themselves whose cellular role is obscure. The nature of these normal components and their relation, if any, to animal viruses is a problem deserving of more investigation.

Up to this point, we have examined the fundamental knowledge derived from investigations of mammalian viruses whose sizes are 40 mμ or larger and whose individual particles can be identified by electron visualization. What have we learned in the last decade about the particles of that group of small viruses which includes the agents of poliomyelitis, the arthropod-borne encephalitides and yellow fever? It is here that the purification of mammalian viruses has made the least headway and the reasons for this are simply stated:

1. As already mentioned, preparations have been contaminated by normal components with properties, such as density and sedimentation, often indistinguishable from the infectious agent.

2. In the case of certain diseases, for example poliomyelitis, the infectivity (and probably the number of particles) is so low that considerable concentration must be effected before sufficient virus is available for study.

3. As concentration and purification proceed, the particles of virus tend to aggregate into masses whose sedimentation and infective levels are no longer those of the individual unit.

4. Finally, in the range of 10 mμ, 100 angstroms, our present electron microscopes are operating only a little above their limits of resolution.

With all respect for the considerable amount of effort expended on the purification of the viruses less than 40 mμ in size, it seems justifiable to state that thus far there has not been much original, or significant, knowledge contributed to the general nature of the animal viruses by these investigations.

Within the past 18 months a method of considerable promise for removing certain of the obstacles mentioned above has been found in the application of protamine precipitation to the smaller mammalian viruses. This procedure is based on the fact that addition of highly basic protamine to infected tissue suspensions will precipitate the tissue debris, and certain viruses are left behind in a clarified supernatant (35). All of the agents reported on thus far have fallen into one of two categories as shown in table 2.

It will be seen that, except for the agent of murine encephalomyelitis, it is the larger viruses which are sedimented while all those which fail to precipitate are 50 mμ or less in size. It should be noted that protamine (salmine) is not virucidal and that its union with virus is a loose one which can be dissolved by re-

TABLE 2

The Behavior of Certain Viruses in the Presence of Protamine

Crude Infected Tissue Suspension	+	Protamine Sulfate	Not Precipitated	Encephalomyocarditis Colorado Tick Fever Coxsackie (Texas) Equine Encephalomyelitis Japanese Encephalitis Poliomyelitis Russian Spring-Summer Encephalitis St. Louis Encephalitis West Nile Virus
			Precipitated	Herpes Simplex Lymphocytic Choriomeningitis Murine Encephalomyelitis Rabies Vaccinia Influenza Tobacco Mosaic Virus

suspension of the precipitate in molar NaCl, an observation first made by Bawden and Pirie (3) for tobacco mosaic virus and protamine (clupein).

The high infectivity and clarity of protamine-treated virus suspensions led to an attempt to sediment out the virus from them by means of ultracentrifugation. When this was done, it was found that highly infectious concentrates from protamine-clarified suspensions had only one-tenth of the nitrogen found in sediments prepared by centrifugation alone. However, any hope that the concentrate obtained from the protamine-treated material was pure virus was dispelled when the same nitrogen content was found in purified sediments from suspensions of normal brain tissue prepared in an identical manner. Attempts at electron visualization of protamine-clarified ultrasediments encountered the same difficulties we have already discussed. So much extraneous debris was present that it was difficult to determine which of the particles was virus.

Recently, the observation has been reported that much of the residual non-viral protein and normal components can be removed with no loss in virus infectivity by digesting partially purified encephalomyocarditis (EMC) virus with crystalline trypsin (36). This procedure, also applicable to other agents, makes satisfactory electron micrography of the smaller viruses possible and enables one to hazard a good guess as to which particle represents the virus. Graphic evidence of this is seen in figures 1–3 (Plate 1).

Figure 1 is an electron micrograph of encephalomyocarditis virus purified and concentrated in the manner described. The larger particles, measuring approximately 30 mμ, are those of the virus. Also seen in this preparation are occasional smaller (18 mμ) particles of a normal mouse brain component. Particles obtained from normal mouse brain are shown in figure 2. Note the extreme homogeneity of the material. The third electron micrograph contains the Texas type of Coxsackie virus obtained from infected mouse brain. The particles measure approximately 24 mμ and it will be appreciated that the differentiation of the virus from the normal component on the basis of the electron photograph is essentially impossible.

In conclusion, we should take cognizance of transitions which are occurring in the approach to the problems of the purification of animal virus. These involve changes in methodology and changes in objective. Our methods have been broadened to include the more utilitarian electric drive ultracentrifuge, chromatography, ion exchange resins, radioactive tracers, replicas for electron microscopy, the micro-complement-fixation test, and digestion with crystalline enzymes. Although the ingenious manipulative methods of the cytochemists are just beginning to be adopted by virologists, more effort is needed in the development of apparatus for handling small amounts of highly infectious purified virus. For example, we need an ultracentrifuge cell capable of direct insertion with its pellet of purified virus into a respirometer or spectrophotometer.

Less obvious are changes in the objectives of current investigation on purified viruses of bacteria, plants, or animals. The arguments of 15 years ago concerning the concepts of molecularity and crystallinity in the animal viruses are no longer paramount. A knowledge of the static physical and chemical properties of purified viruses is obviously insufficient in itself to enable us to understand the essential problems of their metabolism and capacity for self-duplication. In this connection we will do well to heed the admonition of N. W. Pirie, "In studying the mechanism of virus multiplication and its relation to the other processes going on in the cell, it would be a pity if attention were exclusively confined to the bare, flayed residue which can be the result of a successful purification of one of the more stable viruses. It may well be that it is only when this écorché has recovered a skin, by combining with host constituents, that it can manifest its normal activities" (22).

Those engaged in investigating purified animal viruses should ask not only the limited question, "Can we isolate a biochemical aggregate?" but also its corollaries, "Can we isolate a dynamic virus system, and from whence comes its energy?" Let us be encouraged by the opinion of Ralph Waldo Emerson, "The microscope cannot find the animalcule which is the less perfect for being little."

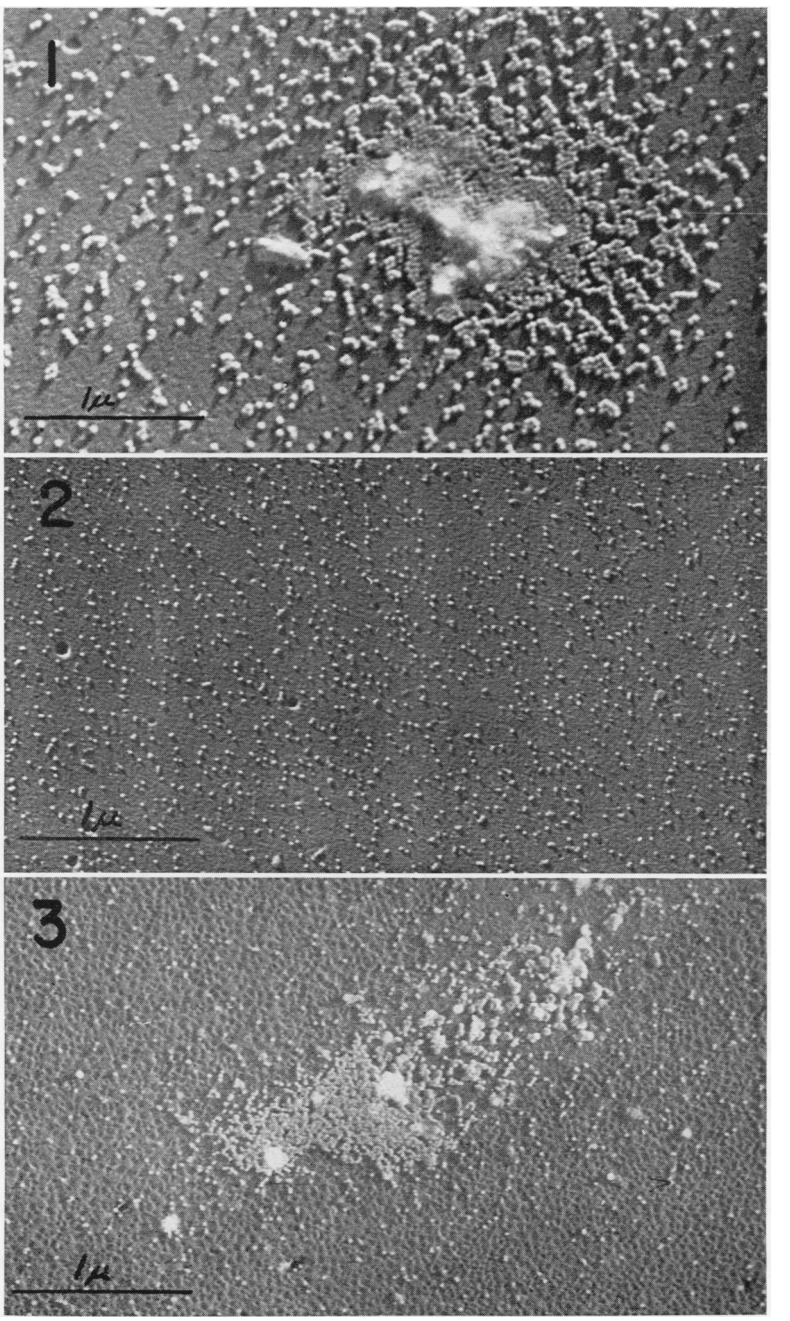

PLATE 1

FIG. 1. Encephalomyocarditis virus from mouse brain. A few particles of normal component are also visible. Electron micrograph, chromium shadowed, magnification 23,400 ×.

FIG. 2. Normal tissue component from mouse brain. Electron micrograph, chromium shadowed, magnification 23,400 ×.

FIG. 3. Coxsackie virus, Texas type, from mouse brain. Normal component is probably present in this preparation. Electron micrograph, chromium shadowed, magnification 23,400 ×.

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PART II

STUDIES ON THE MECHANISM OF REPRODUCTION OF A VIRUS[1]

E. A. EVANS, JR.

Four years ago our research group began a biochemical study of the coli bacteriophages and I have interpreted the invitation to participate in this symposium as an opportunity to describe our results.

It will not be possible for me to do more in the allotted time than summarize the present status of our knowledge and to give some indication of the experiments and methods on which our conclusions are based. Since the reproduction of bacteriophage involves the synthesis of large quantities of nucleic acid and protein, it has been our hope that our results would be of general significance in regard to the nature and interrelationship of protein and nucleic acid metabolism as well as offering information regarding the specific problem of bacteriophage reproduction. I should say immediately that we can only speculate about the more intimate details of the mechanisms concerned. But we do have an increasing fund of information concerning the origin of the various chemical components of the phage particle, and I will be concerned with these data for the major part of my report.

Our experiments have been carried out with the *E. coli* bacteriophage, wild type strain T_6r^+ (9). This can be grown on *E. coli* cultured on a synthetic ammonium lactate medium. Most of our experiments have involved multiple infection of the bacterial host, i.e., the vigorously growing bacterial culture is infected with sufficient phage to insure the adsorption, by each bacterial cell, of at least one virus particle. Under these circumstances, although the oxygen consumption of the host is unaffected, and its assimilation of materials from the medium continues, there is reason to believe that normal protein (2) and normal nucleoprotein (7) metabolism cease and that the metabolic energies of the bacterial cell are occupied chiefly in the synthesis of virus particles. Under these experimental conditions, the yield of phage is high and represents for the most part a single generation of virus.

The purification of the virus is effected by the process of differential centrifugation (9) outlined in table 1. The final product is homogeneous in appearance, has maximal infectivity, and behaves uniformly in the ultracentrifuge and in the electrophoresis apparatus (4, 9, 12). It is possible to prepare amounts sufficient for chemical studies without working on an unduly large and cumbersome scale—from 0.75 to 1 gram of pure virus can be obtained regularly from 10 liters of lysate. Chemical analysis of this material for various components has followed conventional procedures and I shall not discuss these details here.

In tracing the origin of the various components of the phage, we have relied to a large extent on the use of the radioactive isotopes of phosphorus and carbon

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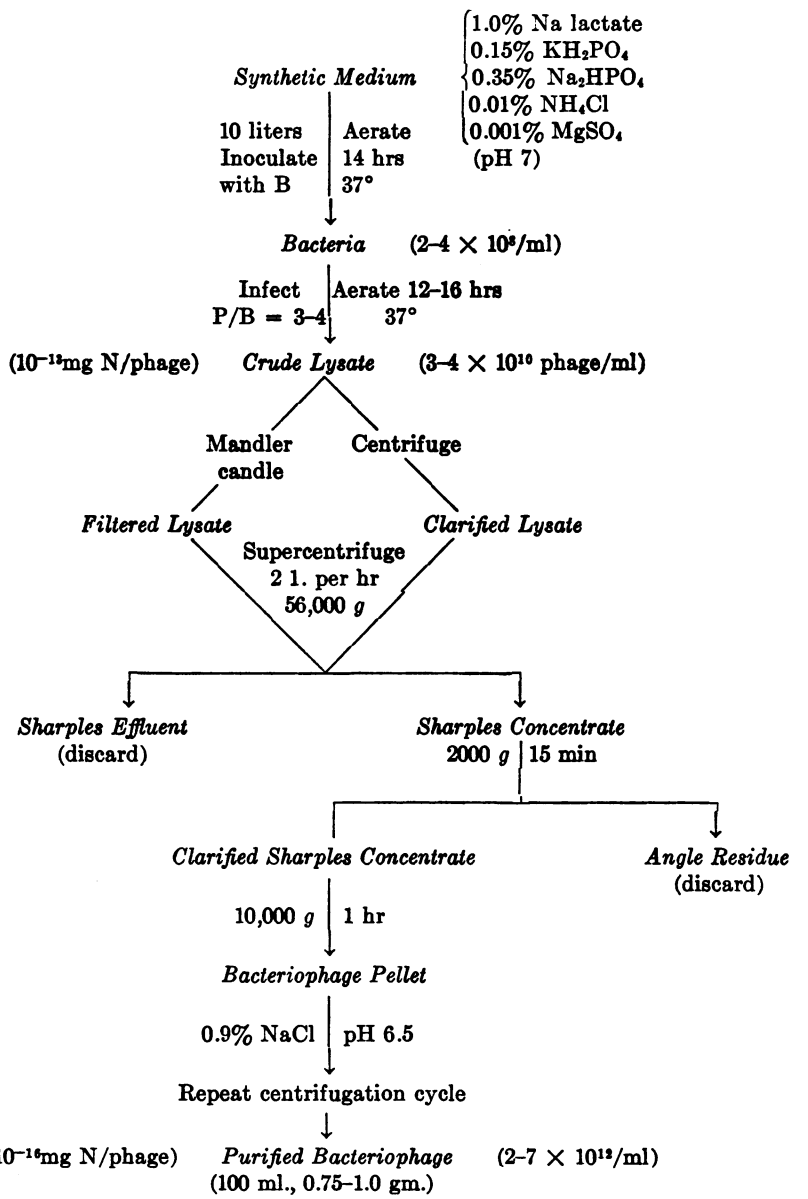
and the heavy isotope of nitrogen. By growing the bacteria on a synthetic ammonium lactate medium of which the phosphate or nitrogen or carbon has been

TABLE 1

Purification of Bacteriophage T_6r^+



replaced, in part, by the proper isotope, it is possible to obtain bacteria labeled in their various component parts. By suspending such bacteria in isotope-free

medium and adding virus, one can follow the transfer of the isotope-marked compounds from the bacterial cell to the virus, the latter being isolated by the procedures already outlined. The reverse type of experiment in which unlabeled bacteria are infected in a labeled medium can also be done, and it is also possible to carry out experiments in which only the infecting virus particle carries an isotopic label. I shall give specific illustrations of these various experimental procedures in what immediately follows.

From a chemical viewpoint, the bacteriophage is roughly half nucleic acid and half protein: of the nitrogen present roughly 50 per cent is present as nucleic acid (predominantly of the desoxypentosenucleic acid type, or DNA); about 40 per cent of the nitrogen appears as protein; and about 6 per cent of the nitrogen is present as the so-called "acid soluble nitrogen" fraction.

Table 2 shows two experiments concerned with tracing the origin of the virus nitrogen; one involving isotope-labeled bacteria in an isotope-free medium, and

TABLE 2

Origin of virus nitrogen
Growth of bacteriophage T_6r^+ on E. coli

	EXPERIMENT I N^{15} Labeled Medium Unlabeled Bacteria		EXPERIMENT II N^{15} Labeled Bacteria Unlabeled Medium	
	Atom % excess	% of N derived from medium	Atom % excess	% of N derived from bacteria
Bacteria, N	0.00	—	9.73	—
Medium, N	10.1	—	0.00	—
Phage, total N	8.19	81.1	2.08	21.4
Phage, nucleic acid N	6.73	66.7	—	27.7
Phage, protein N	9.21	91.2	.76	7.82
Debris, N	2.96	29.3	6.7	69.0

the other involving unlabeled bacteria in an isotope-containing medium. The virus resulting from these cultures was isolated and purified and an analysis made of the per cent of isotopic nitrogen present. It is clear that the bulk of the virus nitrogen has been derived from the ammonia of the medium, and the isotopic label appears in both the protein and the nucleic acid fractions of the bacteriophage. Under these circumstances, large quantities of the protein and nucleic acid that are found in the bacteriophage have been synthesized from the simple components of the medium. We have a series of some fourteen experiments of this type and the data are similar in every case (table 3).

In each instance, however, we find that the bacteria are also making a contribution to the phage N and we have spent considerable time in studying the chemical nature of the material thus being transferred. We cannot say at the present time, with certainty, that we have the complete solution of this problem, but all of our evidence favors the belief that the desoxyribosenucleic acid of the bacterial cell is being transferred in considerable quantities to the virus progeny.

Now the DNA content of the virus produced is more (on the average 2 to 3 times) than is present in the original host so it is clear that DNA must be synthesized during the reproduction of the virus. However, as I have already said, a considerable quantity of DNA is also transferred from the bacterial host to the virus. This is supported by the following considerations.

TABLE 3

Relation of the yield of phage per bacterial cell to the amount of bacterial N contributed to phage protein and nucleic acid

EXPERIMENT*	YIELD OF PHAGE PER BACTERIUM	% OF TOTAL PHAGE N DERIVED FROM HOST	% OF PHAGE PROTEIN N DERIVED FROM HOST	% OF PHAGE NUCLEIC ACID N DERIVED FROM HOST
9	17	38.8	26.6	42.7
14	33	22.1	21.3	16.9
4	50	28.0	21.5	37.2
6	50	28.9	17.6	37.1
10	60	31.1	12.7	43.2
7	80	26.9	17.4	38.1
5	117	20.9	13.2	24.9
13	117	13.1	8.2	16.9
11	117	26.3	10.5	38.2
3	160	20.6	15.1	26.1
8	163	25.9	11.0	37.9
1	173	18.9	8.8	33.3
2	215	21.4	7.8	27.7
12	287	11.6	5.7	16.6

* Arranged in order of increasing yield.

TABLE 4

Kinetic study of the sources of virus nitrogen

	PER CENT OF N DERIVED FROM HOST*					
	Experiment I			Experiment II		
	Incubation time (hours)					
	5.5	7	24	3	5	24
Total phage N	28.9	26.9	25.9	38.3	31.1	26.3
Phage nucleic acid N	37.1	38.1	37.9	42.7	43.2	38.2
Phage protein N	17.6	17.4	11.0	26.6	12.7	10.5

* $\dfrac{\text{Atom \% excess } N^{15} \text{ in virus N}}{\text{Atom \% excess } N^{15} \text{ in bacteria prior to infection}} \times 100.$

In the first instance, we find, in a large series of experiments, that a considerable portion of the virus nucleic acid N is derived from the host and that the quantity involved is independent of the yield of virus (7). Secondly, kinetic experiments of the type shown in table 4 in which the phage from isotope-labeled cells was harvested, purified, and analyzed at the indicated times, show that the

N^{15} content of the phage nucleic acid is independent of the time of liberation of the virus, although the N^{15} content of the phage protein decreases with time and appears to be derived from an exhaustible substrate (6). Similar experiments with bacteria labeled with radioactive phosphorus confirm this view (5, 11). Perhaps the clearest evidence is derived from experiments in which only the purines of the bacterial nucleic acid contain isotopic markers (1). If we grow *E. coli* on synthetic medium supplemented with radioactive sodium bicarbonate, it is possible to isolate from the bacteria purines such as adenine and guanine, and the pyrimidine thymine which contain radioactive carbon. If, now, the pure radioactive adenine is added to another suspension of growing bacteria, it is found that the adenine is incorporated specifically into the purine fraction of the bacterial nucleic acid, partly as adenine, and also after slight chemical modification, as guanine. It is thus possible to obtain *E. coli* in which only the purines, adenine and guanine, of the nucleic acid fraction are labeled with radioactive carbon. When such bacteria are infected with virus and the phage isolated, it is found that the adenine and guanine of the phage nucleic acid are highly radio-

TABLE 5

Transfer of labeled purines from bacterial host to bacteriophage progeny

EXPERIMENT	MATERIAL	THICK SAMPLE COUNT		PER CENT OF PHAGE PURINE FROM HOST
		Bacteria	Phage	
		counts per minute		
I	Purine carbon	4,250 ·	2,230	52
II	DNA adenine	56,500*	7,920*	14
	DNA guanine	59,000*	11,900*	20

* Quantities contained in 10^{12} *E. coli* cells and resulting phage:
Bacterial DNA: adenine 1.08 mg, guanine 1.78 mg.
Phage DNA: adenine 3.68 mg, guanine 2.28 mg.

active and that the isotopic carbon marker appears only in these two compounds (table 5). In other words, a considerable portion of the adenine and guanine of the bacterial nucleic acid have been transferred to the virus progeny along with the large amounts of these two materials synthesized by the bacteria from the medium. In this particular experiment, the virus content of adenine and guanine corresponds to about 60 per cent of the adenine and guanine of the bacterial DNA. In similar experiments with isotopic N as a label, we have found that the N transferred to the virus is equivalent to as much as 85 per cent of the bacterial DNA (7). Since losses always occur under our experimental conditions, it is possible that we might be concerned here with a complete utilization of bacterial DNA for virus synthesis. However, we cannot be certain about this. In other experiments involving bacteria containing the N^{15} labeled pyrimidine, thymine, we observe a similar transfer of this compound to the virus (6). These experiments are of special interest inasmuch as thymine occurs only in the DNA of the bacterial cell (guanine and adenine are components of both types of nucleic acids, DNA and RNA).

Since it appears certain that a transfer of bacterial DNA to the virus does occur, one is concerned with the question as to whether the nucleic acid is being transferred as such, or whether it is broken down into nucleotides, nucleosides, free purines, or other fragments, and then rebuilt. There is evidence that the virus DNA differs in composition (i.e., in the relative amounts of purine and pyrimidine bases present) from the bacterial DNA, but this could result from the transfer of large units of intact bacterial nucleic acid coupled with the synthesis in varying amounts of one or other components of the nucleic acids, for it will be recalled, our data show that the bacterial contribution to virus nucleic acid is supplemented by large quantities of nucleic acids derived from the medium.

In an attempt to study this question, we have carried out experiments in which bacteria were labeled with both isotopic N and radioactive P (6). If a direct transfer of intact nucleic acid from bacteria to host occurred, one would expect to find the ratio $\frac{\%\text{ of virus nucleic acid N from host}}{\%\text{ of virus nucleic acid P from host}}$ to be unity.

TABLE 6

Isotope content of nucleic acid from T_6 bacteriophage grown on E. coli containing N^{15} and P^{32}

EXPT. NO.	BACTERIA		VIRUS NA		RELATIVE ISOTOPE CONTENT		RATIO OF BACTERIAL CONTRIBUTION N/P
	N^{15} atom % excess	Specific radioactivity *c.p.m. per γ P*	N^{15} atom % excess	Specific radioactivity *c.p.m. per γ P*	Virus NA N/Bacterial N	Virus NA P/Bacterial P	
					per cent	*per cent*	
3	9.64	201	3.59	57.4	37.2	28.5	1.31
10	10.5	252	2.74	45.6	26.1	18.1	1.44

However, the observed ratio is from 1.3 to 1.4 (table 6). Evidently then some alteration of the bacterial nucleic acid must occur. The fact that nitrogen and phosphorus are simultaneously transmitted might suggest that at least nucleotide units are involved. Also the fact that the addition of free purines to the medium is without effect on the transfer suggests that more complex units than the simple bases are being transferred (7). The question, however, remains still unanswered.

If the transfer of bacterial DNA is essential to the synthesis of the virus particle, it seems probable that a large unit possessing some degree of biological specificity would be transferred, so that each virus produced would contain an amount of the bacterial DNA fragment. The kinetic experiments which show that the percentage of virus DNA derived from the host is independent of the time of incubation and of the yield of virus would also support such an interpretation. As, in a given experiment, there is the same per cent of isotope in the virus DNA no matter how many virus particles are produced, it seems probable that bacterial DNA is not being used simply as a non-specific and easily available reservoir of materials for the synthesis of those virus particles being formed in the initial stages of virus reproduction, but rather, that each virus

particle receives the same amount of bacterial DNA. We are convinced that the DNA of the host plays an important role in the reproduction of the infecting virus.

As already stated, the virus is roughly half nucleic acid and half protein. We have seen that a considerable portion of the nucleic acid is derived from the bacterial cell and one might ask then whether a similar situation obtains with virus protein. We find, in experiments with N^{15}-labeled bacteria, that a part of the isotope appears in the protein of the phage (6). The most remarkable thing about this contribution of bacterial N to virus protein is that it is so small. The bulk of bacterial protein N is evidently unavailable for virus protein synthesis. I might remark, parenthetically, that the inertness of the protein of infected bacteria has also been noted by Monod and Wollman (8), who showed that phage infected cells were unable to form adaptive enzymes. Also we have found that T_6 infected cells of *E. coli* were unable to form the adaptive enzyme tryptophanase (3). Since it is generally assumed that such adaptive enzymes are formed from other cellular proteins, the failure to form such enzymes upon virus infection suggests that bacterial protein no longer is metabolized in the usual fashion after infection.

The N contributed to the virus protein by the bacteria may be derived from bacterial protein or from some non-protein nitrogenous material other than ammonia. Free amino acids and dipeptides have been found in the acid soluble fraction in quantities sufficient to account for the observed bacterial contribution. It seems plausible to assume that these amino acids and peptides are the precursors of the virus protein rather than that a bacterial protein is being transferred. Since the kinetic experiments show that the percentage of virus protein N derived from the host decreases with yield and time of incubation, and that the protein of phage produced in the terminal phases of the experiment is entirely free from nitrogen derived from the bacterial cell, it seems improbable that there is an obligate and specific contribution of bacterial material to the synthesis of virus protein in contrast to what apparently occurs with virus nucleic acid.

Since the host cell not only contributes material but also supplies the synthetic mechanism for virus reproduction, one naturally inquires whether the infecting particle itself contributes directly to its progeny. We have carried out a number of experiments concerned with this objective and are continuing this phase of the work at the present time. Table 7 contains the data from an experiment in which the virus was labeled with radioactive P^{32} (i.e., in which nucleic acid phosphorus is labeled) and added to a bacterial culture in isotope-free medium (5). The major portion of the nucleic acid phosphate of the infecting particle is liberated during the process of reproduction and appears as unsedimentable low molecular weight phosphorus compounds in the lysate. On the other hand, some 40 per cent of the P of the infecting particle appears in the progeny, probably as nucleic acid. Our supposition is that this represents a specific contribution from the parent particle to each of its offspring and we are now engaged in experiments to establish this conclusion. It is clear, however, that the repro-

duction of the virus involves the destruction of the initial integrity of the infecting particle.

Is it possible, then, to draw any conclusion as to the nature of virus reproduction from the information already at hand? Certainly the fact that the medium is the ultimate source of most of the nitrogen, phosphorus and carbon of the bacteriophage seems incompatible with any idea that the bacterial cell contains a precursor of the virus which undergoes only slight modification during the process of virus reproduction. Likewise the fact that only about 1/12 of the bacterial phosphorus and only about 1/20 of the bacterial nitrogen is used for phage synthesis cannot be reconciled with the suggestion—made largely as a result of a study of electron micrographs—that the protoplasm of the bacterial host is completely utilized in phage reproduction.

However, if it be understood that what I am about to say is to be regarded as of a speculative nature, and as being confined entirely to the reproduction of

TABLE 7

Distribution of radioactivity after multiple infection of E. coli with P^{32} labeled bacteriophage T_6

Protocol: Unadsorbed phage removed by centrifugation. Phage progeny purified by differential centrifugation in angle centrifuge. 60% of virus adsorbed initially. 67% of radio-activity adsorbed initially.

MATERIAL	TITER	RADIOACTIVITY		
	phage/ml $\times 10^{10}$	*cts/min/ml*	*per cent*	*cts/min/phage* $\times 10^{-9}$
Lysate	5.8	84.6	100	1.4
Low speed supernatant	6.2	76	89.8	1.2
High speed supernatant	0.027	41.5	49.2	150
Phage concentrate	85	33.1* (485.2)	39.1	0.57
Purified phage concentrate	120	34.5* (718)	40.8	0.59

* Corrected to original volume. Actual values given in parentheses.

bacteriophage, it would be possible to correlate our present data with a picture such as this. Under ordinary circumstances the bacterial cell manufactures nucleoprotein for its own needs. The synthesizing mechanism involves on the one hand the formation of amino acids, purines and other materials from the simpler compounds of the medium, and on the other hand, the combination of these materials into the larger complexes of protein and nucleic acid. Further, the process is such that the continued production of a specific nucleoprotein involves the participation of this unit in the process itself in such a manner that a portion of the original unit (perhaps a part of its nucleic acid) is incorporated in the replicas that are being formed, i.e., the unique nature of the nucleoprotein being synthesized is a function of the particular fragment of nucleic acid that participates in the process. When a bacterial cell is invaded by a virus, one might speculate that the virus is able to usurp the metabolic machinery of its host only by virtue of the fact that it closely resembles or competes with a normal

metabolic component, i.e., that the virus nucleoprotein would resemble the normal bacterial nucleoprotein-directing element closely enough to occupy the latter's place in the metabolic machinery of the host cell. This machinery, whose function is to manufacture normal nucleoprotein, continues to operate but since the specific nature of its products is determined by the nucleoprotein initiating the reaction, we now have a process in which both the normal bacterial component and the foreign invading particle contribute to and determine the specific nature of the nucleoprotein being synthesized. This is the virus protein which accumulates and leads to the ultimate destruction of the host cell. The host provides the metabolic machinery and energy for synthesizing the virus particle; the particular nature of the virus being formed is determined by specific contributions of nucleic acid from the bacteria and of nucleic acid from the virus itself.

This explanation doesn't differ, I believe, in any marked fashion, from the ideas expressed by others in this field who have been concerned with the genetic and biological aspects of the problem, but I have preferred to express the matter in the simpler chemical terms that are more familiar to me. As I have already said, all of this is speculative at the present time, but the eventual explanation for the mechanism of virus reproduction must conform to the general facts that we have considered this evening.

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PART III

APPROACHES TO THE CONTROL OF VIRAL DISEASES

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The major problems arising in attempts to develop means for controlling viral diseases are not unique and do not differ in any fundamental sense from those presented by other infectious processes. The strategy of the approach is the same, only the tactics are different. Although measures are available for the effective control of but a few viral diseases, each is founded on solidly established principles and has its counterpart in an application useful against certain microbial diseases. That many maladies of viral etiology are not yet controllable can be attributed more to the inadequacy of present knowledge than to the peculiarities of viruses themselves.

As it happens, the enduring immunity which follows infection with any of a number of viruses affords a natural means of limiting the frequency of these ailments and generally restricts experience to a single infection as with many of the common diseases of childhood. Other natural processes, such as genetic background, environmental factors, etc., not yet subject to control because of the limits of knowledge, appear also to play a large role and may serve to keep many viral maladies within bounds. Artificial means of striking efficacy have been devised in exceptional instances, as with smallpox and yellow fever, but in most cases effective control remains only a theoretical possibility.

Artificial control of infectious processes has been accomplished so far by means of three wholly different procedures: (a) elimination of the reservoir of the infectious agent or the means of transmission to the host; (b) specific immunization against the agent or poisons elaborated by it; and (c) therapy with substances of biological or chemical origin which are active against the agent. With a particular infectious disease one of the three classes of control measures may have marked advantages over the others. In many instances none is feasible or useful as yet. With viral diseases as a group this is more generally the case than with any other group of infectious processes. And yet, some of the most highly successful achievements in artificial control, *i.e.*, vaccination against smallpox or yellow fever, have been accomplished with diseases induced by viruses.

Viral disease obviously cannot occur if host-virus association is precluded. This can be achieved in theory in three ways: (a) elimination of the viral reservoir; (b) elimination of viral vectors; and (c) isolation of the host. Ornithosis illustrates the difficulties of handling the reservoir effectively (26); the disease would no doubt disappear if infected birds were destroyed. Far greater difficulties arise in those instances in which man himself appears to provide the reservoir, as seems most likely with many acute respiratory infections, including influenza (20) and the exanthemata of childhood. When the vector is an arthropod, as in the case of yellow fever (35) and dengue (28), means are at hand for its elimination from large geographical areas. The striking success of programs

of this kind for yellow fever (35) are too well known to require further emphasis. Inanimate vectors, whether inhaled, ingested or injected, provide serious problems which have not been solved. It is sufficient to mention the difficulties of air sanitation as bearing on viruses which enter via the respiratory tract or the ridding of blood or products derived from it of hepatitis virus (14) to underline the magnitude of this problem.

Even though host-virus association occurs, when means to prevent it are not available, there are procedures which can prevent cell-virus union. In the absence of such combination it is evident that neither infection nor disease will result. The sole effective procedure of proven usefulness which will accomplish this objective is immunization against the virus in question. The means are again three in number: (a) injection of infective viral vaccine; (b) injection of inactivated viral vaccine; and (c) injection of immune serum. In all cases it is thought that the presence of specific antibody capable of neutralizing the virus is chiefly responsible for the immunity attained.

The classical examples of effective control accomplished with infective viral vaccines are of course smallpox and yellow fever. In both instances a mild viral infection, artificially induced, is employed in order to produce a state of immunity against a severe disease. The striking practical effects obtained with infective vaccines containing either vaccinia virus (31) or the 17D strain of yellow fever virus (35) are directly attributable to the fact that these agents have been developed in a form which is relatively nonvirulent for man; that they induce a persisting immune state; and that the viruses against which they are employed are remarkably homogeneous in antigenic makeup. It may be predicted that whenever all of these properties can be attained with similar vaccines against other viral diseases equally satisfactory control will become feasible.

A number of inactivated viral vaccines have been employed in man but in every case they have yielded results much less satisfactory than those obtained with the infective vaccines mentioned above. Typical examples are vaccines prepared against influenza A and B (4) as well as Japanese B encephalitis (29). With influenza virus vaccine the problem did not at the outset appear sizeable; large quantities of highly concentrated and semi-purified virus are not difficult to obtain; inactivation without much loss of immunogenic power is readily accomplished; neutralizing antibody responses upon injection of the vaccine are about equivalent to those resulting from the disease itself. Two features have caused trouble; the transience of the immunity induced (19) and the remarkable variation of the antigenic constitution of influenza viruses (30). Vaccines containing inactivated viruses may be useful for essential personnel at critical periods, but the relatively short duration of the immunity they give seriously limits their effectiveness, and makes necessary regularly repeated injections with all the hazards this entails.

The injection of appropriate immune serum could in theory prevent, abort or modify almost any viral disease if sufficient specific antibody were given early enough after entry of the infective agent. Only in rare instances is appropriate

immune serum available and usually there is no information as to the nature of the infection which is in incubation. So far application of this control measure has been made almost exclusively in the case of measles (32) with results which are well known. The use of human immune serum is not free of risk and the possibility of unwittingly inducing viral hepatitis (14) is ever present.

Although not of proven efficacy in man, there are additional means by which cell-virus union might be prevented. Stemming from investigations with bacterial viruses (5) and certain animal viruses which cause hemagglutination (17), the concept of cell receptors has evolved. Substances which clearly alter the influenza virus receptors of erythrocytes (3) have given some indication of altering host cells susceptible to infection with these agents (33) although the effect has been of brief duration (6). In another direction attempts have been made to find substances which could stand as chemical analogues of the cell receptor and compete with it for the virus (2, 36). Although a number of such substances (1, 18, 34), highly active *in vitro* with the influenza-mumps group of agents, are known none has given much promise of a useful effect *in vivo* under practicable conditions.

When neither host-virus association nor cell-virus union can be prevented, as appears at present to be the usual situation, there seems to be no recourse but to fall back on therapy as a means of control. Effective therapy of viral diseases in all probability will come to depend upon procedures which can inhibit or interrupt viral multiplication, a process which is thought to occur within the host cell (21). Inhibition of viral multiplication has been accomplished in experimental infections by means of two procedures: (a) viral interference; and (b) chemotherapy (23).

Interference between viruses is a startling phenomenon which if fully understood might shed light both on the mechanism of viral multiplication and on means to interrupt it. Present evidence supports the idea that interference is a competitive phenomenon dependent upon alterations caused in host cell metabolism (15). Although the procedure has, as yet, no useful application in the control of viral diseases in man, its efficacy is a theoretical possibility. Both infective and appropriately inactivated viruses can cause interference with the multiplication of certain other viruses and may do so when the infectious process is under way, as has been shown with influenza viruses (16, 37). Although this altered state is transient and seldom persists for more than a week, the effect is striking. Given an agent relatively nonvirulent for man, available in high concentration and adequate supply, effective control might be feasible by substituting a mild or inapparent infectious process in place of a more severe illness. Not all pairs of viruses show reciprocal interference, however, and the choice of an appropriate agent for such a purpose would require considerable skill.

Chemotherapeutic substances have been available for two viral diseases, lymphogranuloma venereum (13) and trachoma (25) since 1938. Substances useful against all maladies induced by the psittacosis-lymphogranuloma group of viruses are now known and include sulfonamide drugs as well as various antimicrobial substances such as penicillin, aureomycin and chloromycetin (23). In this con-

nection, it may be of importance that the viruses of this group are the largest known, probably are complex in organization and constituents and may be similar to rickettsiae in metabolic requirements. In addition, it has been reported that the course of herpes zoster (7) or primary atypical pneumonia (27) was somewhat modified by administration of aureomycin. In both instances additional carefully controlled studies appear desirable. Despite vigorous and extensive investigations, chemotherapeutic substances effective against the bulk of viral diseases of man have not yet been found.

The principles underlying the chemotherapeutic approach to the control of viral diseases have been studied in considerable detail with two experimental models. The multiplication of either pneumonia virus of mice or mumps virus can be interrupted upon the injection of highly purified polysaccharides derived from Friedländer bacilli (10, 24). Multiplication of other medium or small size viruses is unaffected by these substances. A single injection of a few micrograms, in either the mouse or the chick embryo, is sufficient to restrict multiplication to a very small percentage of the concentration attained in control animals. With either virus, the polysaccharide is effective when one injection is given 4 days after inoculation. Both viruses have a latent period of approximately 20 hours and then show a sudden increase in concentration closely analogous to the single burst of bacterial viruses. After massive inoculation and the simultaneous infection of large numbers of susceptible cells, polysaccharide is effective in interrupting viral multiplication when given during the first half of the latent period (9). This is strong evidence in support of the concept that the substance interrupts the intracellular process upon which multiplication depends. Additional evidence favoring this idea is derived from the finding that the polysaccharide has no effect upon either virus *per se*; that adsorption by susceptible cells is unaffected by high concentrations of the substance (11); that a variant obtainable from large populations of mumps virus is wholly resistant to the inhibitory effects of the substance (12).

The structural configurations of the polysaccharide molecule which endow it with this remarkable property are not yet known. The molecule may be altered by drastic chemical procedures, which cause it to lose all serological activity, without affecting its inhibitory capacity (10, 24). Dialysable fragments obtained by hydrolysis are, however, inactive. There is as yet no direct information pointing to the nature of the host cell system or component which is affected by the substance. Indirect evidence raises the possibility that the cell component is not present in large amount and is not rapidly formed since a minute amount of polysaccharide exerts a marked effect for 10 days or more (24). Because highly active quantities of the substance cause no evidences of toxicity, it seems probable that the cell component is not essential to survival of the cell. That availability of this cell component is not necessary for the multiplication of all viruses is deduced from the finding that the polysaccharide has no effect on the multiplication of influenza or Newcastle disease viruses (10).

If the polysaccharide is to be considered as a chemotherapeutic agent effective in controlling an experimental disease induced with a small virus, it should exert

a clear effect after gross evidence of disease is present. Recent findings indicate that this is the case. When the substance is given long after inoculation, at a time when viral multiplication is proceeding at maximal rate and gross pneumonia is already present, it interrupts any further multiplication and correspondingly inhibits progress of the pneumonic lesion (8). Under these circumstances administration of the substance converts an overwhelming infectious process which kills all controls into a modified disease from which animals recover.

Such control of viral diseases as has been achieved till now has depended upon the application of one or another of those procedures which are also effective in controlling other infectious processes. It seems doubtful that the eventual control of viral diseases will require means fundamentally different from those already devised even though the operative mechanisms may differ in important details (22). In a few instances viral diseases of man can be controlled by precluding the possibility of host-virus association through elimination of the vector, in some instances control is feasible by preventing cell-virus union through specific immunization, and with one group of large viruses a measure of control can be achieved with chemotherapeutic substances. Various mechanisms relating to each of these procedures appear open for exploration and it may be that their exploitation will lead to the possibility of far broader control than is now attainable.

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PART IV

THE DENGUE GROUP OF VIRUSES AND ITS FAMILY RELATIONSHIPS[1]

ALBERT B. SABIN

The filtrability of the etiological agent of dengue fever, first reported by Ashburn and Craig (1) in 1907, was subsequently confirmed by a number of other investigators. By the time the important monograph of Simmons, St. John and Reynolds (10) was published in 1931, a great deal of information, laboriously gathered by studies on human volunteers, had become available on the behavior of the virus in human beings, mosquitoes and certain monkeys. When my own studies on dengue began early in 1944, little was known about the basic properties of the virus, about its immunologic and immunogenic characteristics, and no source of virus was available for reference or study other than that which might be obtained from patients with the disease. I have had an opportunity to carry out comparative studies on seven different strains derived from patients in Hawaii, New Guinea and India, and on three mouse-adapted strains kindly supplied by Japanese investigators after the war. The purpose of this communication is to present a brief summary of what has been learned thus far of the biologic and immunologic characteristics of the dengue viruses as they occur in nature, of the changes resulting from continued propagation in mice, and of the antigenic relationships with the viruses of yellow fever, West Nile fever, and Japanese B encephalitis.

Biologic Characteristics of Dengue Viruses Recovered from Patients and Maintained by Passage in Human Volunteers. A filtrable agent can be included in the dengue group of viruses when, in addition to reproducing the typical clinical picture of dengue in human volunteers, it can also be transmitted by *Aedes aegypti* mosquitoes after a suitable extrinsic incubation period. When a strain of virus identified in this manner is available for reference, other strains can also be identified by immunologic comparison without resorting to the mosquito transmission test. The reliability of this method of initial identification of the seven human strains, which I studied, was confirmed when it was found that none of three strains of sandfly (phlebotomus) fever virus recovered from patients in the Mediterranean region was either transmissible by *Aedes aegypti* or immunologically related to the dengue group. Another important property of the dengue group of viruses is the capacity to produce a skin lesion at the site of intracutaneous injection. All seven strains of dengue virus and none of the three strains of phlebotomus fever virus produced such a lesion. The specificity of the local skin reaction was also established by neutralization with homologous convalescent serum. Human dengue virus has a size of 17 to 25 mμ as determined by gradocol membrane filtration. The various strains have proved to be

[1] The work discussed in this communication was part of the Army Medical Department's program under the Armed Forces Epidemiological Board.

highly stable on storage in the frozen state at about −70 C, and in the lyophilized state at about 5 C for at least 5 years, and are available for reference and comparative study.

Human dengue virus produces an inapparent infection in rhesus monkeys after intracerebral as well as after intraperitoneal injection. Neutralizing and complement-fixing antibodies appear between 14 to 21 days after inoculation and persist for many months. Focal infiltrative lesions of the type seen in nonparalytic poliomyelitis have been found in the spinal cord of monkeys sacrificed six weeks after intracerebral injection of human dengue virus (5). An essentially inapparent infection has been produced in chimpanzees, which also developed neutralizing and complement-fixing antibodies (4) that persisted for at least ten months (5). Human dengue virus is not pathogenic for rabbits, guinea pigs, hamsters or cotton rats. It could not be propagated in embryonated eggs or in cultures containing minced chick embryo, mouse embryo or human leukocytes (6). Human dengue virus injected intracerebrally in suckling or older mice only rarely gives rise to clinical manifestations of infection. However, such mice are resistant to inoculation of the highly virulent mouse-adapted virus, during the first days as a result of interference, and during the subsequent months as a result of active immunity.

Characteristics of Mouse-Adapted Dengue Virus. Although all human strains of dengue virus apparently multiply after intracerebral injection in mice, only one of our strains, the Hawaii, developed sufficient virulence for the mouse on continuous serial passage to permit its use in serologic and immunogenic experiments (8). It required 15 serial passages before all mice inoculated intracerebrally with the maximum amount of virus exhibited clinical evidence of infection, consisting in almost all instances of flaccid paralysis. During the course of further passages the titer gradually rose to a level of 10^{-5} to 10^{-6} with corresponding shortening of the incubation period. At the present time after 114 serial passages, the titer still depends on the age of the mice in whose brains the virus is propagated. If the mice are one to seven days old at the time of inoculation, their brains yield virus suspensions with an intracerebral titer of 10^{-7} to 10^{-8} upon titration in 2- to 3-week old mice. However, even after many serial passages in newborn mice, the titer reverts to 10^{-5} to 10^{-6} after a single passage in 14-day old mice. The use of newborn mice for routine passage with the resulting titers of 10^{-7} to 10^{-8}, led not only to the development of potent complement-fixing antigen (9) but also to more satisfactory tests for neutralizing antibody.

The mouse-adapted Hawaii virus differs from the unmodified human virus in the following respects:

1. It has lost the capacity to produce the febrile systemic illness in human beings, but still produces the rash.

2. *Aedes aegypti* feeding on human beings inoculated with 15th mouse-passage virus failed to transmit the infection even after prolonged extrinsic incubation periods. This, however, may be due to very low titers of virus in the circulating blood rather than to an inability of the modified virus to grow in the mosquitoes.

3. In human beings it gives rise to neutralizing antibodies and complete im-

munity to infection with unmodified virus, but not to complement-fixing antibodies.

4. In rhesus monkeys intracerebral injection of 1×10^5 to 1×10^7 mouse LD_{50} regularly produces a febrile illness, occasionally followed by typical flaccid paralysis of the extremities. A fatal paralytic disease, which clinically and pathologically is not readily distinguishable from experimental poliomyelitis, occurs more frequently with virus of greater potency derived from newborn mice (5). Both neutralizing and complement-fixing antibodies develop in these monkeys, but the C-F antibodies are of lower titer than in monkeys inoculated with human virus and disappear after six weeks.

5. The thoroughly mouse-adapted virus can be propagated in embryonated eggs while the human virus cannot.

The mouse-adapted Hawaii virus has remained nonpathogenic for cotton rats, hamsters, guinea pigs and rabbits. In mice it behaves as a predominantly neuronotropic virus.

Thirty-three human volunteers inoculated with varying amounts of mouse-adapted virus showed no signs suggestive of involvement of the nervous system (6, 8), but no tests have as yet been carried out with material which has had more than 19 mouse passages. The paralytic effects produced on intracerebral inoculation in monkeys appear to be the result of the greater number of mouse LD_{50} of virus injected rather than any qualitative change in the virus. However, further studies on this question might be indicated before the more virulent mouse-passaged virus is used in human beings.

During the war a number of Japanese investigators reported on the pathogenicity of dengue virus in mice and other laboratory animals. The broad host range and other properties of the viruses reported as dengue by Ishii (2) and by Yaoi and Arakawa (12) are in marked contrast to the properties just described. My own studies on these strains indicated that the virus reported by Ishii was actually that of Rift Valley Fever, and the Kimura strain submitted by Yaoi and Arakawa a strain of fixed rabies. However, the three mouse-adapted strains (Mochizuki, Sota and Kin-A), which were kindly supplied to me by Kimura and Hotta of Kyoto University, turned out to have the same host range and immunologic properties as the Hawaii virus. Furthermore, neutralization tests carried out in my laboratory with the convalescent sera of patients who had the disease during the large wartime epidemics in Japan, indicated that the epidemics were caused by a virus of the Hawaii type (5).

Immunologic Relationships within the Group of Dengue Viruses. The existence of at least two immunologic types of dengue virus was established by (a) active cross immunity tests in human volunteers, (b) dermal neutralization tests with convalescent sera in human volunteers, (c) neutralization tests in mice with convalescent sera from human beings and rhesus monkeys, and (d) complement-fixation tests with convalescent sera of human beings and rhesus monkeys.

Human volunteers reinoculated with the same strain of virus proved to be completely immune 18 months after a single infection. These tests are especially significant because they were carried out in nondengue areas and there can be

no question of the immunity having been reinforced by intercurrent, inapparent reinfection. The results of reinoculation with a heterologous strain depend upon the interval after the original attack. During the first one to two months there is active immunity to heterologous as well as to homologous strains. That this effect is most likely due to a common antigen and not to nonspecific resistance resulting from a preceding febrile illness is confirmed by the fact that phlebotomus fever convalescents exhibit no such immunity to dengue virus. From two to nine months after the initial attack, reinfection with a heterologous virus usually results in a modified form of the disease which is of shorter duration, less severe, and without rash. By this method of comparison it was found that four of the seven human strains studied, i.e., the Hawaii, New Guinea "A" and two strains from India, belonged to one group or type, while the other three, all from New Guinea, belonged to another.

Dermal neutralization tests in human volunteers were carried out by inoculating mixtures consisting of nine parts of undiluted normal or convalescent

TABLE 1

Differentiation between strains of dengue virus

(Dermal neutralization tests in human volunteers)

STRAIN OF VIRUS	SKIN LESIONS RESULTING FROM MIXTURE WITH					
	Normal serum	Human convalescent serum				
		Hawaii	"N.G." A	"N.G." B	"N.G." C	"N.G." D
Hawaii	+	0	0	+	+	+
New Guinea "A"	+	0	0	+	+	+
New Guinea "B"	+	+	+	0	0	0
New Guinea "C"	+	+	+	0	0	0
New Guinea "D"	+	+?	+	0	0	0

dengue serum with one part of acute dengue serum diluted to contain approximately ten minimal skin-lesion-producing doses. The results obtained in tests with five human strains of virus, shown in table 1, reveal the existence of only two immunologic types—the Hawaii and New Guinea "A" belonging to one type and the New Guinea "B," "C" and "D" strains to another.

Neutralization of the mouse-adapted dengue virus by intracerebral tests in mice has been found to depend on two factors: (a) specific antibody which is heat stable (56 C for 30 min) and (b) a nonspecific, complement-like, heat-labile accessory substance which produces the maximum inactivation of the sensitized virus after incubation *in vitro* for two hours at 37 C. Thus, when the dengue virus had an intracerebral titer in mice of only 10^{-3} to 10^{-4}, no neutralization was demonstrable either when heated serum was used or when fresh (or frozen) serum was used without *in vitro* incubation of the serum-virus mixtures. When fresh (or frozen) serum was used and serum-virus mixtures were allowed to incubate for two hours at 37 C, convalescent sera usually yielded neutralization indexes of 100 to 300. After the virus had reached an intracerebral potency of

approximately 10^{-7}, the neutralization indexes obtained with homologous convalescent sera were in the range of 10,000 to 100,000. The effects of either heating the serum or eliminating the incubation period, and the role of the nonspecific, heat-labile accessory factor are shown in table 2. By means of the mouse neutralization test carried out under optimum conditions with virus of high potency, it was found that neutralizing antibodies for the Hawaii type of virus persist in high titer for at least four years (the longest period tested thus far) after a single experimental infection in human beings residing in nondengue regions (5). The neutralizing antibodies are type-specific. The majority of human volunteers or rhesus monkeys, inoculated with the New Guinea "B," "C" or "D" strains of human dengue virus fail to develop a significant titer of antibodies for the Hawaii mouse-adapted virus; an occasional individual and a few rhesus monkeys, however, yielded sera with neutralization indexes 100 to 1,000 times lower than those found after infection with the homologous strains of virus. By means of these neutralization tests it was found that seven of ten strains of dengue virus belong to one immunological type; these seven strains are the Hawaii, New Guinea "A," two strains from India, and the Mochizuki, Sota and Kin-A mouse-adapted strains isolated by Kimura and Hotta (3) in Japan.

TABLE 2

Role of heat-labile nonspecific accessory factors in neutralization of dengue virus

(Neutralization tests carried out by intracerebral route in 3-week old mice; intracerebral titer of virus used varied from $10^{-6.5}$ to $10^{-7.4}$

EXP.	SERUM USED (UNDILUTED)	DILUENT FOR VIRUS (UNDILUTED *normal* SERUM)	INCUBATION OF MIXTURE	NEUTRALIZATION INDEX
I	*Antidengue* rhesus—*frozen*	Rabbit—*heated*	37 C, 2 hours	50,000
	" " —*heated*	" — "	" " "	160
II	" " —*frozen*	" — "	None	500
	" " — "	" — "	37 C, 2 hours	50,000
	" " —*heated*	" — "	" " "	200
	" " — "	Guinea pig—*heated*	" " "	130
	" " — "	" " —*frozen*	" " "	50,000
	" " — "	Human—*heated*	" " "	630
	" " — "	" —*frozen*	" " "	13,000
	Normal rabbit—*heated*	" — "	" " "	1
	" " — "	Guinea pig—*frozen*	" " "	1
	" rhesus—*frozen*	Rabbit—*heated*	" " "	1

The dengue complement-fixing (C-F) antigen of choice is a benzene-extracted preparation made from the brains of newborn mice inoculated with the Hawaii mouse-adapted virus (9). Human volunteers and rhesus monkeys infected with human strains of the Hawaii type or with the heterologous New Guinea "B," "C" or "D" strains develop C-F antibodies for the Hawaii antigen. In human volunteers inoculated with the heterologous New Guinea strains, the C-F titers at two months were in the range of 1:4 to 1:16 as compared with titers of 1:64

to 1:256 among those infected with the Hawaii type viruses. At six months, however, several volunteers infected with the New Guinea "B," "C" and "D" strains no longer had C-F antibody for the heterologous Hawaii virus, while all of seven volunteers who had received the Hawaii viruses were still positive at three and four years after a single infection, with titers ranging from 1:2 to 1:128. Rhesus monkeys inoculated with the heterologous human dengue viruses exhibit the highest titers (as high as 1:256) for the group-specific C-F antibody at three to four weeks, with practically complete disappearance at six weeks in some animals and longer persistence in others. A few examples of the results obtained with

TABLE 3

Complement-fixing and neutralizing antibodies for Hawaii dengue virus in human beings naturally infected in different parts of the world

REGION	TIME AFTER KNOWN ATTACK	PATIENT	ANTIBODIES FOR HAWAII DENGUE	
			Neutralizing	Complement-fixing
Hawaii	2 years	Par.	+	1:64
		Nit.	+	1:32
		Tak.	+	1:32
Osaka, Japan	1 year	Kis.	+	1:16
	2 years	Yas.	+	1:8
		Mor.	+	1:32
Guam (American marines)	11 months	S-1	−	1:8
		S-2	−	1:8
		S-3	−	1:2
		S-4	−	1:8
		S-8	−	0
		S-10	−	0
New Guinea (American officer)	5 years	Sil.	+	1:2
Singapore (Japanese scientist)	6 years	Sas.	+	1:32
Dutch East Indies (Dutch scientist)	18 years	Din.	+	1:64

convalescent sera obtained from natural cases of the disease contracted in various parts of the world are shown in table 3.

Relationships of the Dengue Group to Other Viruses. The relationship with the virus of yellow fever was the first to be investigated because of similarity in size and mosquito vectors. In human volunteers the 17D strain of yellow fever commonly used for vaccination, when administered a few days before or simultaneously with unmodified human dengue virus, prolonged the incubation period of the resulting dengue, and greatly lessened the severity and duration of the illness. This effect was obviously due to an interference phenomenon since, when the interval between administration of the yellow fever and dengue viruses was

prolonged to 35 days, there was no immunity to as little as ten minimal infective doses of dengue virus, and the resulting illness was not modified in any way. In experiments carried out in association with Dr. Max Theiler, a similar interference phenomenon between the dengue and viscerotropic yellow fever viruses was also demonstrated in rhesus monkeys and *Aedes aegypti* mosquitoes (11). However, a definite antigenic relationship between these agents was demonstrable by the complement-fixation test. Rhesus monkeys receiving a single intracerebral injection of human Hawaii or heterologous New Guinea dengue viruses developed no neutralizing antibodies for the yellow fever virus, but at three to four weeks after inoculation all exhibited C-F antibodies with titers ranging from 1:8 to 1:32 for the yellow fever antigen and 1:128 for the Hawaii virus antigen. The same rhesus monkeys also *developed* C-F antibodies for the West Nile and Japanese B encephalitis viruses with titers ranging from 1:16 to 1:64, but not for the St. Louis, Western equine or Rift Valley fever viruses. It should be stressed that these group relationships by the C-F test were demonstrable only with the most potent 20 per cent, benzene-extracted, brain antigens, suggesting

TABLE 4

Complement-fixing antibodies for various viruses in American volunteers experimentally infected with Hawaii type human dengue virus

STRAIN OF VIRUS AND MODE OF INFECTION	VOLUNTEER	TIME AFTER INFECTION	C-F TITERS WITH INDICATED VIRUSES							NORMAL MOUSE BRAIN
			Hawaii	Jap B	West Nile	Yellow Fever	SLE	WEE	Rift Valley	
"Calcutta" Human serum	Spe.	2 months	64	4	8	4	—	0	0	0
"Hawaii" *Aedes aegypti*	Bay.	7 months	128	8	8	4	0	0	0	0

that the common antigenic groups were present in much lower concentration than the type-specific antigenic component. A few of these monkeys also developed neutralizing antibodies for the Japanese B and West Nile viruses with neutralization indexes ranging from 20 to 500. American volunteers, never out of the U. S. A., also exhibited C-F antibodies for the Japanese B, West Nile and yellow fever viruses after experimental infection with human dengue virus either by the bite of *Aedes aegypti* mosquitoes or injection of human serum (table 4).

This antigenic inter-relationship between the dengue, yellow fever, West Nile and Japanese B encephalitis viruses was also demonstrable by C-F tests with antisera for these other viruses, but it should be pointed out that with few exceptions positive results were obtained only with potent hyperimmune sera. Thus, the most potent Japanese B mouse hyperimmune serum had the following C-F titers: 1:256 for Japanese B, 1:128 for West Nile, 1:32 for yellow fever, 1:4 for dengue, 1:2 for St. Louis, and nothing for the Western equine and Rift Valley fever viruses. All rhesus *hyperimmune*, yellow fever antisera (kindly sup-

plied by Dr. Max Theiler) with homologous C-F titers of 1:32 to 1:128, had C-F antibody for the West Nile and Japanese B antigens in titers of 1:4 to 1:32, but only six of twelve such sera had C-F antibody for the dengue virus with titers of 1:4 to 1:16 (5, 7).

The demonstration of a common antigen for this group of viruses has a bearing not only on the interesting aspects of a possible generic relationship between the dengue, yellow fever, West Nile and Japanese B encephalitis viruses, but also on the design and interpretation of serologic tests for diagnostic and epidemiologic studies.

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PART V

THE POLIOMYELITIS, ENCEPHALOMYOCARDITIS, AND COXSACKIE GROUPS OF VIRUSES[1]

JOSEPH L. MELNICK

The task of the current reviewer has been lightened, as far as poliomyelitis is concerned, by several excellent summaries which have appeared in 1949 (15, 17). As a consequence, I shall deal only with those aspects of poliomyelitis which I feel have not been adequately brought together and upon which new experimental data have been brought to bear. In particular I shall discuss the host-virus relationship as it occurs in the preparalytic phase or in the nonparalytic disease after natural routes of infection.

In the laboratory this problem can be most satisfactorily studied in animals which become infected through administration of virus by peripheral routes. This infection need not necessarily be one of the CNS, a point which has been underscored by the work of Enders on the growth of poliomyelitis virus in non-nervous human tissues. If monkeys are fed poliomyelitis virus, for example, serum antibodies may appear quickly whether or not paralysis develops (21, 22). In fact when paralysis did occur after feeding virus, antibodies were found on the first day that paralysis was observed, in contradistinction to the delayed antibody development in monkeys paralyzed following inoculation of virus by the intracerebral route. This early antibody development also occurs in man, where, if one uses the strain producing infection in the patient, antibodies may be found to be present at the onset of paralysis and to increase in amount during the next few weeks (8, 18).

The chimpanzee seems to be the animal of choice for studying the virus-host relationship in the nonparalytic infection. Following oral exposure or intradermal inoculation of virus these animals respond by becoming intestinal carriers of the virus and by developing neutralizing antibodies. In some instances humoral antibodies may even be found before detectable amounts of virus appear in the stool. By following the response of such chimpanzees to repeated exposures with the same and different immunological types, it has been possible to gain some insight into the development of immunity (9, 10). If such animals, once infected with a certain strain, are fed again the same type of virus, it will pass through the alimentary tract as though it were an inert substance, and subsequently no carrier state will appear. However, if a different antigenic type of virus is fed, then the response is like that of a new animal. The animal becomes a carrier of the new type of virus and develops antibodies to it.

It is probable that this is the experience of man also. Available statistics suggest that second paralytic attacks occur in persons who have had poliomyelitis

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at about the same frequency with which first attacks occur in the normal population. Recently Bodian and Howe (2) were able to recover virus from a poliomyelitic patient who had had a paralytic disease, presumably poliomyelitis, fourteen years earlier. During the second paralytic attack, Leon type virus was isolated and the patient developed antibodies to this type which persisted for at least 5 years. We have also had occasion to study a second attack, in which several members of a family were involved (24), (table 1).

In October, 1944, poliomyelitis came to the B family living in a rural area about ten miles from New Haven. The oldest child, aged 15, died. Her spinal cord had typical poliomyelitic lesions and was pooled with 3 others from fatal cases which occurred in the same area that summer, and from this pool poliomyelitis virus was isolated. At the time of the fatal illness, a sister, Belle, aged 13, developed paralytic poliomyelitis, and four other siblings had illnesses compatible with nonparalytic poliomyelitis. Virus studies were not made on the siblings.

TABLE 1

Second attack of poliomyelitis in the B family

AGE	B FAMILY	OCT., 1944 ILLNESS	6 JUNE, 1946		20 JUNE, 1946	OCT., 1946	MAY, 1947	JULY, 1947
			Illness	Virus	Virus	Virus	Virus	Virus
15	Frances	Fatal polio						
14	John	Headache	Fatal polio	+				
13	Belle	Paralytic polio	None	+	+	−	−	−
10	Norma	Headache, stiff neck	None	+	−	−	−	−
8	Florence	Vomiting	None	+	+	−	−	−
6	Rosalie	Vomiting, diarrhea	None	+	+	−	−	−
48	Father	None	None	−	−			
34	Mother	None	None	−	−			

In June, 1946, fatal poliomyelitis returned to the B family. John, now 16, died of poliomyelitis and at the time of his illness all four of his sisters were intestinal carriers of the virus. Two weeks later three of the girls were still excreting virus, including Belle, the child who suffered a paralytic attack in 1944 and who still in 1946 had signs of the after effects of her first illness. None of the other children was ill in 1946 at the time when poliomyelitis returned to the family. Unfortunately no information is at hand concerning the types of virus which caused the illnesses in 1944 and in 1946. If we assume that these types were different, then the response was similar to that of the chimpanzees. An alternative possibility is that the immunity following the 1944 attacks had worn off by 1946.

The multiple cases of simultaneous infection with poliomyelitis which apparently occurred in this family are similar to those reported by others. Brown and Ainslee (3) have recently expanded the study of poliomyelitis in families to include, in addition to virus tests, antibody determinations both to the Lansing type and the type responsible for the family outbreak. Their results show that

the healthy children in the family respond precisely as does the patient (or patients) not only in respect to virus excretion but also in respect to the development of antibodies to the current family strain. The fact that antibodies to the family strain appear in most of the siblings at the same time as they appear in the patient emphasizes again that families appear to be exposed as an entire unit. It is of interest that at the time of onset of illness in Brown and Ainslee's patients, the adults had antibodies both to Lansing and to the family type whereas the children had antibodies to the family type alone. Furthermore, the adults were not carriers of the family virus as were the children. A rational explanation of these findings seems to be that the adults had had prior infections with both

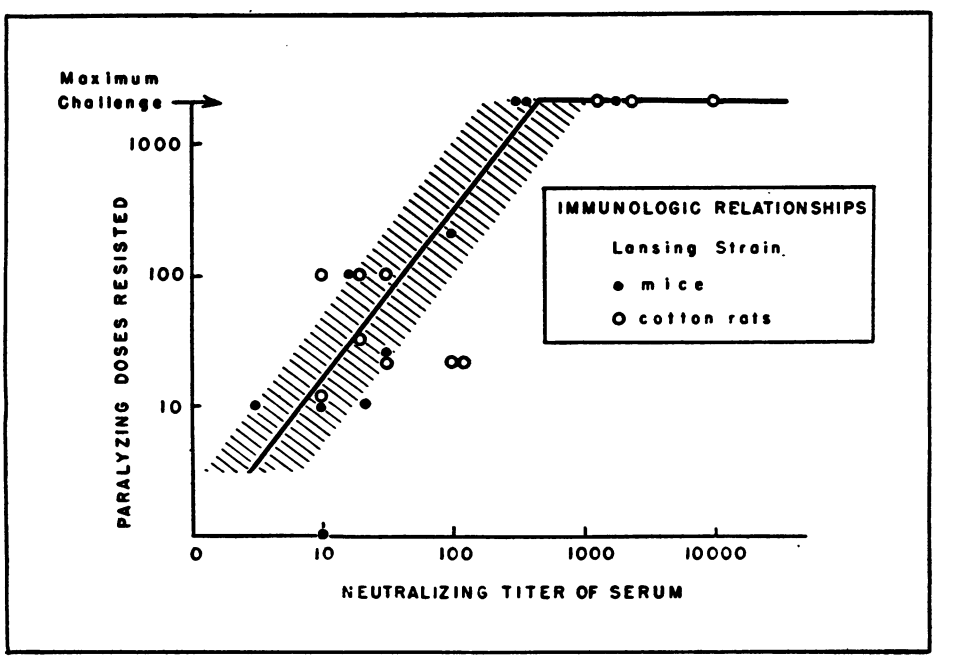

FIG. 1. Relationship in cotton rats and mice between neutralizing titer of serum and the amount of paralyzing doses of virus resisted when the latter challenge dose was administered intracerebrally.

the Lansing and family types, and were immune to both at the time of the family type outbreak. If this interpretation is true, we should expect to find in the period immediately following the occurrence of a case, increases in antibody titers to the family types but only in the children who were currently infected with this type, and not in the adults who supposedly had been previously exposed and made immune (in the fashion of the chimpanzees cited above).

This brings us to the question of persistence of immunity in poliomyelitis. First, can we say that the lvel of antibody is correlated with the immune status? From experiments in laboratory animals this appears to be so. For example, when groups of mice and cotton rats were immunized with varying amounts of virus to yield varying antibody levels as shown in figure 1, direct correlation was

found between the concentration of antibody present and the ability of the animal to withstand infection following intracerebral challenge (24). As the antibody titer increased, both species of animals were able to withstand higher doses of virus until at an antibody level of 1:500 and above they were solidly immune, and could withstand the maximum challenge dose of 2000 ID_{50} which was set by the infective titer of the virus in these animals. Similar data for monkeys have been obtained by Morgan (14).

We found that antibodies and immunity persisted together in our chimpanzees, for at least two years. If we assume that the presence of antibodies may be an index of the immune status, then perhaps we can, for the present, support the view that one infection produces a lasting immunity to the strain causing the infection. Paul and Riordan (16) have recently carried out a series of neutralization tests using the Lansing strain with over 200 sera collected in 1949 from remote Eskimo villages in northern Alaska (near Point Barrow). The early results are as yet unpublished, but I have been permitted to quote that they have found Lansing strain antibodies to be present almost exclusively in those aged 20 and above. From the scanty records available, it would appear that an epidemic of poliomyelitis was present in this area in 1930, but there is no knowledge of its presence before or since. The serological results may be interpreted to indicate then that the Lansing type of poliomyelitis virus had been present in 1930, but not since. This observation is, of course, no more remarkable than the finding of yellow fever antibodies in people who, following an attack of the disease, have not lived in endemic or epidemic areas for over 50 years. The lack of Lansing antibodies in Eskimos under 20 is in marked contrast to the early appearance of antibodies in people in other parts of the world.

The position of another virus group which for a time was confused with poliomyelitis virus has recently been elucidated by Warren (23) and Dick (7). This group is distinct from Theiler's TO and FA types of spontaneous mouse encephalomyelitis virus and as yet only one immunological type has been discovered. This virus, for which the name encephalomyocarditis, or EMC, has been suggested as being descriptive of the pathological lesions in experimental animals (23), was discovered in 1940 by Jungeblut (12). From wild cotton rats inoculated with the Yale- or Y-SK strain of poliomyelitis virus, he recovered a virus with antigenic structure, host range, and pathogenic properties different from the original Y-SK strain. Attempts to define this agent as a poliomyelitis virus were confusing to other workers because Jungeblut, in his reports, had not excluded the possibility that this "new" virus had been adventitiously transferred from naturally infected rodents into which the Y-SK virus had been inoculated. The MM strain (which is related immunologically to Columbia SK) may also have been derived in similar fashion in the process of passaging human CNS into a hamster in the Columbia laboratory. A cooperative study has been undertaken between Dr. Jungeblut and our laboratory to unravel some of the mystery of the origin of the Columbia SK strain.

After Jungeblut's original report of the Columbia SK virus, he found that

when he passed Y-SK virus, which had been adapted to mice in the Yale laboratories and which in every way fulfilled the criteria of a poliomyelitis virus (4), the titer occasionally increased markedly from its usual level, leading him to suspect a contamination with another virus or else a mutation. Consequently, when he found that immune sera prepared against Y-SK virus which had been propagated in mice in his laboratory neutralized both Y-SK and Columbia SK viruses, he and I were not satisfied that this was proof for the derivation of Columbia SK from Y-SK. New experiments were set up in which the Y-SK virus was propagated in mice at Yale. The infected CNS was then harvested and used to immunize monkeys in our laboratory and part was sent to Dr. Jungeblut to be used to immunize monkeys at Columbia. Neutralization tests were carried out both at Columbia and at Yale with concordant results. The results obtained in New Haven are shown in table 2. Antiserum to the Y-SK virus prepared at Yale neutralized only the Y-SK virus. Antiserum to Columbia SK virus prepared at Columbia neutralized only the Columbia SK virus. Y-SK antiserum

TABLE 2

Antigenic differences between Yale SK and Columbia SK

IMMUNIZING VIRUS	VIRUS PROPAGATED IN MICE AT	ANIMALS IMMUNIZED AT	SOURCE OF SERUM	LOGS OF VIRUS NEUTRALIZED		
				Columbia SK		Yale SK
				IP† titer, $10^{-7.8}$	IC† titer, $10^{-7.9}$	IC titer, $10^{-3.5}$
Yale SK.....	Yale	Yale	Monkey	0	0	>3.0
Yale SK.....	Yale	Yale	Chimpanzee*	0	0	>3.0
Yale SK.....	Yale	Columbia	Monkey	>1.8	1.9	>3.0
Columbia SK.....	Columbia	Columbia	Monkey	>4.8	1.9	0

* After oral infection.

† IP indicates mice inoculated intraperitoneally; IC, intracerebrally.

prepared at Columbia (using Y-SK virus propagated at Yale) neutralized *both* Y-SK and Columbia SK viruses. My interpretation must be that the two viruses are different.

The recovery of the related EMC virus from a chimpanzee that died in Dania, Florida, in 1944, and the observations by Warren of antibodies in rats trapped in the area several years later, has led to the suspicion that the origin of the Columbia SK virus may have been in the Florida cotton rats into which the Y-SK strain had been passed. Recently another member has been added to the group, the Mengo encephalomyelitis virus (7) which was recovered in 1947 in Africa from man, monkey, mongoose and mosquitoes. Some of the properties of this important group of viruses, for whose discovery we are indebted to Dr. Jungeblut, are listed in table 3.

Another group of viruses with which those of us in the poliomyelitis field have had to deal is the Coxsackie group. Originally isolated by Dalldorf from the stools of patients on whom a diagnosis of poliomyelitis had been made, it

seems that infection with these agents may mimic such diseases as nonparalytic poliomyelitis, aseptic meningitis, epidemic myalgia or pleurodynia, influenza, summer grippe, or simply fever of unknown origin. The picture is further complicated in that a patient may have a simultaneous infection with two distinct agents. Although only two years have elapsed since the discovery of the agent, a considerable amount of information has been amassed and has recently appeared in review form (5, 6, 13). We are now in a position of knowing more about the properties of the Coxsackie or C viruses than about the illnesses which they cause, which is an unusual state of affairs in the history of infectious disease.

TABLE 3

Family relationships in the EMC group

	COLUMBIA SK	MM	ENCEPHALOMYOCARDITIS	MENGO ENCEPHALOMYELITIS
Isolation	1940, New York (Florida rat?)	1943, New York (Columbia SK Lab.)	1944, Florida Chimpanzee	1947, Uganda, Man, Monkey, Mongoose, Mosquitoes
Size	10 to 15 mμ (pass 30 mμ filter)			
Host range	Mouse, hamster, cotton rat: rapidly fatal encephalomyelitis Guinea pig: fever with or without paralysis Monkey: occasional transient paralysis (viremia 4th–15th day) Albino rat, rabbit: inapparent infection Chick embryo			
Pathology	Diffuse polioencephalomyelitis of entire CNS (cerebral and cerebellar cortex) Acute, interstitial myocarditis (myonecrosis and inflammatory cell reaction) Intracerebral passages → death in 2–3 days with no cardiac lesions Intraperitoneal passages → death in 7 days with cardiac lesions			
Antigenic relationships proved by	(a) cross neutralization tests (b) cross complement fixation tests (c) cross immunity tests			

The group of C viruses may be divided into two subgroups which Dalldorf calls A and B, but which probably represent two families of viruses. Group A is made up of at least five immunological types which characteristically produce in mice a flaccid paralysis, with myositis as the chief and usually only lesion. In accordance with the pathological findings, the virus is found in highest titer in the muscle, with a relatively high ratio of concentration of virus in the blood to that in the muscle. Group B consists of at least two immunological types which produce in mice spastic paralysis and tremors, although flaccid paralysis may also occur with these types. The pathological findings are widespread with several tissues being involved, particularly the fat pads and the brain. The virus is found

in low titer in the blood, and the titer in muscle is often not much higher than that in the brain and other tissues such as the liver and intestines. Certain other properties of the virus are listed in table 4 in which similar properties are listed for poliomyelitis virus.

Members of the Coxsackie group of viruses were first isolated from patients ill during the summer of 1947 and subsequently numerous recoveries have been made both in this country and abroad (1, 5, 6, 11, 13, 19, 20).[2] Members of this group have also been isolated from sewage and flies collected during 1948 and 1949 (13). It is of interest that a C virus strain, immunologically related to strains recovered in Texas and North Carolina in 1948, has recently been isolated from flies collected during the 1943 epidemic of poliomyelitis in New Haven. Besides showing that the virus may be kept stored on dry ice for over six years, this finding demonstrates that the virus was in existence several years before its discovery, and that the strain isolated belongs to a common type (Texas). In addition to the isolation of the Texas type from flies trapped in the lower Rio Grande Valley in 1948 and from sewage collected the same summer in High Point, North Carolina, this strain was recovered from patients in Dallas, Texas, in 1949 (19). Serological evidence, *i.e.*, presence of neutralizing and complement-fixing antibodies in normal sera, also indicates that the Texas type is widespread, existing in several areas of this country as well as in Denmark and northern Alaska (24).

The specificity of the serological response to different types of C virus is demonstrated by studies recently carried out on 6 patients who accidentally contracted their disease in the laboratory. Parenthetically, it should be noted that this unfortunate incident proves that three different immunological types of C virus (one, Texas, belonging to Group A and two, Connecticut and Ohio, to Group B) are capable of causing human disease. In each case virus was isolated from the patient, and an increase in the antibody titer to the homologous strain was observed soon after the onset of the disease. The data on four of the patients are shown in figure 2. They illustrate that at the time of increase of antibodies to the homologous strain there was a similar response to the prototype to which the homologous strain was related. Patient G. J. exhibited a response only to the homotypic strains, and antibodies to two other types were not present during this period. Patient N. L. already had Ohio strain antibodies when she started work in our laboratory and these were maintained for the nine month period of study. However, some time between February 10 and May 26 she apparently contracted a subclinical infection with the Texas type. The virus isolated at the time of her illness, onset on May 21, fell into the Connecticut

[2] In view of the frequency with which Miss Howitt (11) has been able to recover C virus from her normal mice when blind passage was carried out, I do not feel qualified to interpret her results. In our laboratory we have been unable to isolate the virus from the acute phase sera of over 20 patients from each of whom virus was recovered in the stools and from over 40 patients suspected of C virus infection but from whom virus was not isolated. In regard to the observation of finding C virus in human CNS obtained from fatal cases of poliomyelitis, no mention was made as to the precautions taken at autopsy to avoid contamination of this tissue.

TABLE 4

Comparison of properties and behavior of poliomyelitis virus and C virus

	POLIOMYELITIS VIRUS	C VIRUS
Virus Properties		
Number of known immunological types	3	7
Resistant to		
Ether	+	+
Penicillin	+	+
Streptomycin	+	+
Chloromycetin	+	+
Heat inactivation (aqueous medium for 30 min.)	50–55 C	60 C
Sedimentation Constant (Svedberg units, S_{20})	120	120
Filtration Diameter (mμ)	8–17	15–23
Experimental Disease		
Mouse:		
Susceptibility:		
Virus titer in newborn	Y-SK, $10^{-1.5}$	10^{-5} to 10^{-8}
Virus titer in adults	$10^{-3.6}$	0 to 10^{-3}
Virus distribution	CNS	Muscle, CNS, feces, blood, etc.
Chief Lesion	Myelitis	Myositis
Monkey:	Myelitis with paralysis	Fever (?), pharyngeal & intestinal carriers
Antibodies after feeding virus	+	+
Chimpanzee after feeding virus:		
Apparent disease	− (occ. +)	−
Viremia	?	+
Pharyngeal carrier	?	+
Intestinal carrier	+	+
Early antibody rise	+	+
Homotypic immunity	+	+
Heterotypic susceptibility	+	+
Chick Embryo	−	−
Tissue Culture (human and mouse embryo, respectively)	+	+
Interference with poliomyelitis virus		−
Interference with C virus	−	
Natural Disease in Man		
Virus in throat	+	+
Virus in stools	+	+
Virus persists longer in stools than throat	+	+
Neutralizing antibodies present early (at onset of disease)	+	+
Complement fixing antibodies	?	+
Clinical features:		
Fatalities	+	?
Paralysis	+	?
Pleocytosis in CSF	+	+
Stiff neck and back	+	+
Chest and abdominal pain (epidemic myalgia)	−	+

TABLE 4—*Continued*

	POLIOMYELITIS VIRUS	C VIRUS
Epidemiological Factors		
Family outbreaks	+	+
Summer disease	+	+
Virus found in sewage	+	+
Virus found in flies	+	+
Antibodies in normal gamma globulin and in normal sera	+	+

group. Patient E. W. S. had a similar story, except that he never had antibodies to the Ohio type during the study. The results of tests on patient J. L. M. proved of interest because of the six serial bleedings obtained over the course of a year while he was actively engaged in work on several types of C virus. He became ill on May 18. Serum of that day contained no antibodies to the homologous strain isolated from his stools or to the homotypic Connecticut strain. By May 26, such antibodies were present in titers of 1:250 and 1:100 respectively, and the prototype Connecticut antibody level was maintained through October 20. Antibodies to the Ohio type were present at the time of the first bleeding, November 29, 1948. There may have been an anamnestic response of Ohio type antibodies at the time of illness with Connecticut virus, for the titer of Ohio strain antibodies rose during the first week of illness from a baseline of 1:100 in November, 1948, and 1:10 in February, 1949, to 1:100 on May 18 and 1:1000 on May 26. It fell to 1:100 on June 12 and remained at this level through October 20. It may be noted that the Connecticut and Ohio types belong to Group B. Infection at the subclinical level with Group A Texas type occurred in this individual between November, 1948, and February, 1949. There was no increase in Texas antibodies following the acute illness in May. Perhaps antibodies to Group A strains are not subject to recall at the time of Group B infections, or perhaps the supposed *anamnestic* phenomenon observed with B group viruses may have been more apparent than real.

Simultaneous excretion of both C virus and poliomyelitis virus from the intestinal tract of man both during periods of illness and apparent health (1, 6, 13) and the simultaneous recovery of these two viruses from flies trapped in poliomyelitis areas (13), have made it important to establish whether the host is infected by both agents or whether one is merely in passive transit through the alimentary tract. This answer is needed before one can consider whether the symptoms of the patient represent a single or combined infection. In the course of an investigation of the severe 1949 poliomyelitis epidemic in Easton, Pennsylvania, samples were collected by Dr. N. W. Larkum from 48 consecutive patients entering the hospital for poliomyelitis. From these we isolated poliomyelitis virus *and* C virus from 15 patients, and of the latter, two paralytic patients were selected for further study.

The acute phase stools of each patient were found to contain poliomyelitis virus in a titer of 10^{-2} in monkeys. This enabled us to use this original human

source material as the virus in the neutralization test. The C virus titer of these stools in newborn mice was $10^{-1.0}$ and $10^{-3.0}$, respectively. Tests were set up by mixing 10^{-1} concentrations of stool with varying dilutions of serum, and the mixtures, after incubation, were inoculated into monkeys and into newborn mice.

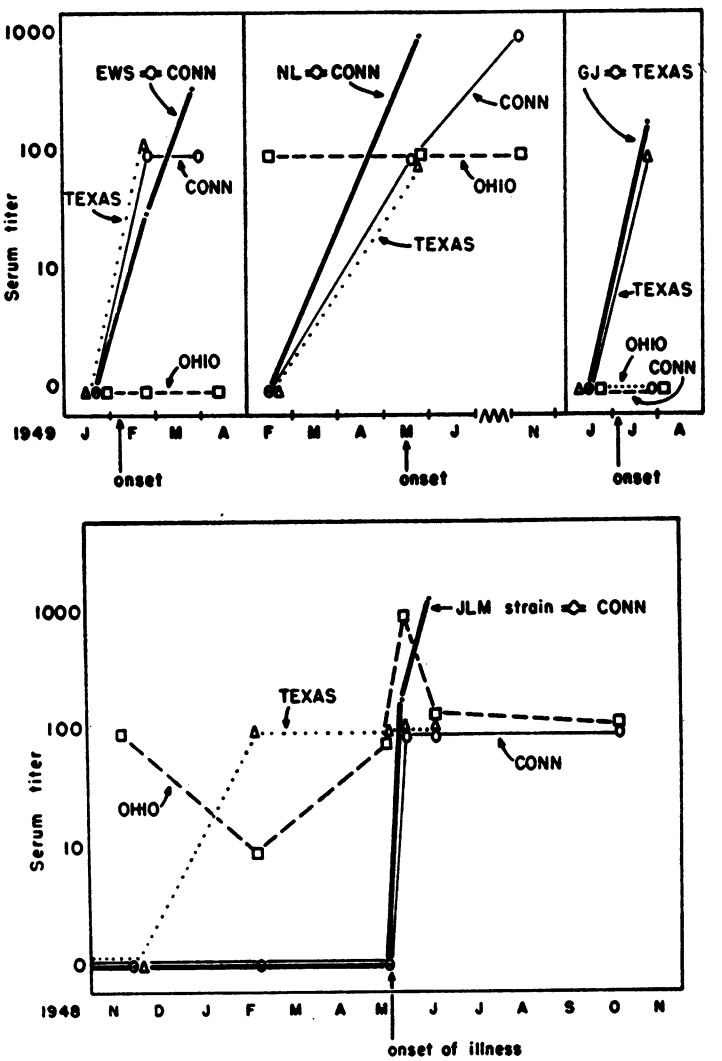

FIG. 2. Laboratory infections with C virus. For each of the 4 patients, the time of onset is shown in relation to the antibody response to 3 prototype strains of C virus (Texas, Ohio, and Conn No. 5) as well as to the strain isolated from the patient.

Both patients responded to their illnesses by simultaneously developing antibodies to poliomyelitis virus and to C virus. Results obtained using mouse passage virus confirmed the increases of antibodies to C virus found when the original human virus was used. As has been reported for both poliomyelitis virus

and C virus, antibodies for each of the viruses were already present in the acute phase and both antibodies rose together within the next four weeks.

With these results we are forcibly brought back into the poliomyelitis problem. We believe that the failure to detect poliomyelitis virus in the stools or throat of a paralytic patient excreting C virus should not necessarily be regarded as an indication that C virus caused the lesion resulting in paralysis, and that for the present and until it is proved otherwise, we should consider the patient's paralytic manifestations to be the result of CNS infection with poliomyelitis virus. However, the finding of dual infections in poliomyelitis appears to occur too frequently to be regarded merely as coincidence. Is it possible that infection with poliomyelitis would have been a mild affair in these patients had not C virus infection been superimposed on the poliomyelitis infection? Consideration is being given to the possibility that C virus infection may be one of the predisposing factors which turns a nonparalytic into a paralytic case.

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PART VI

1. RICKETTSIALPOX AND Q FEVER

ROBERT J. HUEBNER

Rickettsialpox and Q fever are acute, infectious diseases caused by specific unrelated rickettsiae, which in recent years have been found to be indigenous to the United States. Both entities occur essentially as infections of lower animals. Man serves as an accidental host and appears quite unnecessary for the maintenance of either rickettsial agent in nature.

RICKETTSIALPOX

Rickettsialpox was first described in 1946 as a result of studies which were initiated following a request from the New York City Health Department for the National Institutes of Health to assist in an investigation of an outbreak of a strange illness occurring in a large housing development in the borough of Queens. Observations of more than 100 ill persons revealed several unique features which were inconsistent with such diagnoses as chickenpox, Rocky Mountain spotted fever, Brill's disease, infectious mononucleosis, and meningococcemia, which were frequently used by physicians to describe the disease.

Featured during the prodromal stage of 85 per cent of the cases was a papulovesicular lesion accompanied by a regional lymphadenopathy (17). Three to ten days later a remittent fever occurred, often spiking as high as 104 to 105 F but lasting less than a week. Within 24 to 36 hours after onset of fever, a generalized non-itching papulo-vesicular rash appeared. The individual lesions of the rash, like the initial lesion, began as small erythematous papules which, as they increased in size, acquired a centrally located vesicle. Unlike the initial lesion, which persisted as an eschar for several weeks, the generalized rash usually disappeared shortly after defervescence. A transient enanthem on the palate and the tongue was noted in a few cases. No complications or fatalities occurred. Except for leucopenia, usual clinical laboratory studies generally gave negative results. Routine serologic tests were not helpful. Prior and subsequent observations by others were fully consistent with this clinical picture (70, 54, 48, 49, 1).

When it became obvious that this was indeed a previously undescribed disease, a systematic study was undertaken by the New York City Health Department and the National Institutes of Health with the important assistance of a pest control operator and a number of private physicians. First of all several acutely ill persons were hospitalized and studied carefully from a clinical and pathological standpoint. At the same time, blood, urine and biopsy tissue specimens were taken during the acute stage of illness of 20 patients, and injected into nearly all types of experimental animals which were available at the National Institutes of Health. Blood specimens from two of these persons produced objective illness and death in mice. In three weeks a strain of rickettsial organisms was established in mice, guinea pigs and in the yolksacs of chick embryos (27). The disease was then named rickettsialpox because of the distinctive poxlike skin

lesions manifested by the human disease. The newly isolated rickettsia, named *Rickettsia akari*, was found to possess the physical and biological attributes of other known rickettsiae, to grow well in yolksacs, and to furnish a useful complement-fixing antigen. Immunological studies indicated that it was related to but not identical with other rickettsiae of the spotted fever group.

Epidemiological studies indicated that age, sex, occupation, school attendance, food and water supplies did not influence the occurrence of cases of rickettsialpox in the housing development (16). However, the remarkable sparing of the surrounding community suggested that the housing development harbored the source of the infection. Mice were observed in the apartments but early examinations for parasites were unsuccessful. Fortunately, at the very moment that the laboratory studies of human materials suggested a rickettsial etiology, the presence of an unusual mite, *Allodermanyssus sanguineus*, in the households of cases was brought to our attention. A field laboratory succeeded in collecting large numbers of these mites. At the National Institutes of Health, six strains of *Rickettsia akari* were isolated from separate pools of *A. sanguineus* and this mite was shown to be capable of transmitting *R. akari* to suckling mice by feeding on them (25). House mice (*Mus musculus*) which were trapped in the same housing development during the field laboratory studies were shown to be naturally infected with *R. akari*, as well as infested with the mite, *A. sanguineus* (21). Epidemiological surveys of mites in relation to the presence of rickettsialpox cases indicated that an association existed between them. Basement storerooms and apartment house incinerators were found to be heavily infested with mice and seemed to be important factors in the spread of this disease in New York City.

Subsequent studies by the New York City Health Department, and others (12, 48, 49) have resulted in the isolation of additional strains from human beings and have established Manhattan and the Bronx as the chief contemporary sites of human infection with rickettsialpox; several hundred cases have been reported from these areas in the past two years. Rickettsialpox has not been definitely recognized outside of New York City up to the present time. However, systematic searches for this disease in other urban areas have not been carried out. Aureomycin may be of value in treatment (49). To summarize, it would appear that rickettsialpox is essentially a murine disease caused by *R. akari* and under suitable circumstances transmitted to man through the agency of the rodent mite, *A. sanguineus*.

Q FEVER

Q fever is an acute specific rickettsial disease of man which is characterized by sudden onset, high fever, headache, malaise and a pneumonitis. Severity and duration varies considerably. Complications and sequelae are not uncommon but only nine deaths have been reported.

For eight years following its initial description in 1937 by Derrick, Q fever in man appeared to be confined largely to livestock and abattoir workers in Australia. However, during this period laboratory studies of *Coxiella burnetii*, the

causative agent of Q fever, were quite fruitful, and field studies demonstrated the organism in humans, bandicoots and ticks. Other animals, particularly cattle, were incriminated as potential reservoirs but *C. burnetii* was not isolated from them.

Interest in Q fever as a disease of man was greatly stimulated by reports in 1945 of outbreaks among American, British and German troops in Italy and some of the Balkan states, and reports in 1946 of outbreaks in livestock and packing house workers in the United States. Studies of these widespread outbreaks served to reaffirm the Australian observations which indicated (a) that person to person transmission was unimportant, and (b) that most cases were associated with some form of animal life, principally livestock.

Unfortunately all these studies[1] were by necessity retrospective and provided poor opportunities for demonstrating *C. burnetii* in potential sources of infection.

In 1947 when Q fever was found to be endemic in southern California, contemporary and continuous studies of Q fever were finally made possible in this country. These and similar studies in other areas in the United States (35, 4, 69, 28, 29, 47) and in Europe (68, 40, 19, 7, 18, 10, 44) during the past three years have extended considerably our knowledge of the distribution and behavior of *C. burnetii* as a naturally occurring parasite of man and certain domestic animals.

Initial investigations of cases (72, 55, 2) and subsequent large scale epidemiological surveys (3) in the Los Angeles area of Southern California revealed that many thousands of persons had been infected with *C. burnetii* in recent years and that these infections produced definite illnesses which were sometimes serious and occasionally fatal. Each of these investigations focused particular attention upon dairy cattle as the most likely sources of human infection in that area.

Intensive studies of dairy cattle, dairy products, and dairy environments revealed the following:

(a) An appreciable proportion, exceeding 10 per cent, of many thousands of dairy cows in the Los Angeles area milkshed were shown to be infected with *C. burnetii*. Furthermore, it was estimated that 40 to 50 per cent of all uninfected immigrant dairy cows became infected within six months after being shipped into this area (26). Although the majority of cows with positive reactions soon gave negative responses, many remained chronically infected for periods exceeding two years.

(b) Large numbers of Q fever organisms were present in the milk of infected cows, and as a consequence of this the organism was demonstrated continuously in the raw milk supplies of the majority of the larger dairies tested in the Los Angeles milkshed (26, 23).

(c) Pasteurization greatly diminished but did not completely eliminate the comparatively resistant *C. burnetii* from milk (24).

[1] Several recent publications contain excellent reviews and adequate bibliographies on the general subject of Q fever (56, 22, 14, 42). Consequently, the chief subject of this paper will be a brief summary of the results of the Southern California studies which were carried out as a cooperative project by the U. S. Public Health Service, The California State Dept. of Public Health and the Health Departments of the County and City of Los Angeles.

(d) Parturient placentas of infected cows were shown to contain as much as 100 million infectious doses for guinea pigs per gram of tissue (39). Frequent parturitions and high rates of infection indicated that such materials were rich sources of contamination to the dairy environment, in contrast to urine and feces of such naturally infected cows in which *C. burnetii* was not demonstrated despite many attempts (26).

(e) The tissues of *Otobius megnini* taken from the ears of cattle were shown to contain *C. burnetii* (30). This tick however was not observed to attack man and its possible role in the spread of *C. burnetii* to cattle was not defined. Studies of other arthropods were entirely negative.

(f) Although *C. burnetii* was demonstrated in sheep and goats (as previously shown by workers in Greece and northern California (35, 7), field investigations did not suggest that these species were comparable to dairy cows as potential sources of human infection in this area (3, 26, 31).

The results of epidemiological investigations of human infections in the endemic area of Southern California can be divided into those obtained in studies of frank cases and those obtained in large scale epidemiological surveys of specific population groups for infection and disease. The initial study of 300 cases (2) showed that unusually large proportions of patients had occupations in livestock industries, lived near dairies, or used raw milk in their households. These case studies alone, however, were not sufficient to establish the natural prevalence of the disease, since the case finding methods used could not guarantee that the sampling was representative enough to give an accurate impression of the actual occurrence of Q fever.

Consequently, epidemiological and serological surveys of specific population groups selected so as to represent various degrees of contact with dairy cattle and their products were made in order to obtain more accurate information (3). Nearly 10,000 persons in the Los Angeles area and more than 2000 persons from other areas were surveyed. Exhaustive personal and household histories were supplemented by complement fixation tests of blood serums for antibodies against *C. burnetii*. A comparatively insensitive complement fixation technique was used. However, the test was shown by epidemiologic study to be highly specific for recent infection with *C. burnetii*. Most of the groups which were surveyed contained sufficiently large numbers of persons to permit tests of significance of the associations which were found to exist.

The results of these large scale surveys were not only consistent with the observations of the previous case study but provided additional evidence that Q fever infections in the Los Angeles area were most apt to occur in persons who used raw milk in their households, in persons with residence near dairies, and in persons with occupational exposure to livestock or raw livestock products.

To summarize, Q fever was shown to be a well established endemic disease in Southern California and to have caused many thousands of illnesses in recent years. Local dairy cattle and some of their raw products were found to be the most frequent sources of human infection and disease in that area.

2. NEWER KNOWLEDGE OF THE OLDER RICKETTSIAL DISEASES

EDWIN H. LENNETTE

During the past 10 years, the addition to our knowledge of the rickettsial diseases, and of their causal agents, has been so great that when confronted by restrictions of time and of space, one finds it difficult to select one subject over others of equal interest or importance. In the present instance, we have chosen to discuss the epidemiology of Q fever because of the current interest in this disease, and to mention some of the recent developments and trends in prophylaxis, a subject always of general interest. Finally, some discussion of chemotherapy is included because recent additions to the antibiotic armamentarium have provided for the first time highly effective weapons for the treatment of rickettsial diseases.

EPIDEMIOLOGY OF Q FEVER

Some aspects of the epidemiology of Q fever have already been presented in this symposium. We should like, however, to discuss the somewhat different epidemiologic picture that we have observed in Northern California.

In this portion of California, which includes in general the 48 counties lying north of the Tahachapi Mountains, more than 400 proved human cases have occurred during the past two years. Over two-thirds of the cases developed during the months of March, April and May; very few occurred during the summer or fall months. The higher incidence of the disease in males is a striking feature; about 10 times as many males are affected as females.

Cases have occurred in persons representing a wide age group, but the majority have been in males of the active working-age group of 20 to 49 years.

While patients with Q fever have been encountered in 20 of the 48 counties of Northern California, the greatest number were found in a few primarily agricultural counties in the Great Central Valley. Here, in addition to an endemic pattern of incidence, there have appeared several small localized outbreaks. For example, approximately 60 cases of Q fever occurred in students of an agricultural college over a period of a few weeks, 40 cases were observed in a small agricultural community in a similar period of time, and 11 human infections occurred over a short period in a small abattoir.

At the time our studies were begun, the recorded literature pointed to a relationship between contact with cattle and subsequent development of human infection. Therefore, in the field investigation of the early cases, particular attention was given to the possibility that exposure to livestock had occurred. It was soon evident, in these early cases, that while there was a history of exposure to domestic livestock, the animals implicated were sheep and goats rather than cattle (36).

Serologic examination of the sheep and goats with which these patients had been associated revealed that a variable, but high, proportion (up to 86 per cent

of sheep, 75 per cent of goats (35)) possessed complement-fixing antibodies to *Coxiella burnetii*.

As more and more cases were uncovered and investigated, a history of exposure to both sheep and cattle was obtained. In these instances, serologic studies on both species with which patients had been associated uniformly showed that the proportion of serologically positive sheep was high, the proportion of serologically positive cattle low (less than five per cent (35)).

Because of these findings, it was considered necessary to obtain, for comparative purposes, data on the prevalence of complement-fixing antibodies in the general livestock population. For this purpose, blood specimens obtained from abattoirs were used. It was thus found that among the general cattle and sheep population, i.e., among animals with no known association with human cases, only three per cent possessed demonstrable complement-fixing antibodies to *C. burnetii* (35). This low prevalence of antibodies in the general livestock population implies that the finding of a high proportion of serologically positive animals is an uncommon event, and suggests that a high prevalence rate when present indicates the existence of an epidemiologic relationship between such animals and the associated human infection. In support of this, in Southern California, a high proportion of dairy cattle associated with human cases possess antibodies; in Northern California, on the other hand, a high proportion of the sheep and goats associated with human cases have antibodies. However, in at least one instance in Northern California, a group of cases had a history of association with dairy cattle (34); in this instance, complement-fixing antibodies were found in a high proportion of the cows, and the rickettsiae were isolated from the milk of some of these animals (34).

While most patients in Northern California had a background history of exposure to domestic livestock, many did not. Of those who gave no such history, a certain proportion may actually have been exposed, directly or indirectly, since they resided in areas where the raising of livestock is a major occupation. In at least one group of cases in an urban area, there was no good evidence of direct contact with livestock.

It thus appears that while contact with livestock may be an important factor in the epidemiology of human infection, not all cases of Q fever can be adequately explained on such a basis, and other possible sources and means of infection must be considered. In Northern California, no good evidence has been adduced that milk, water, food, or arthropods are commonly involved, if at all, in the transmission of Q fever to man. Also, there is evidence that person-to-person transmission is not important (34).

One of the factors that was considered early to be of possible importance in the epidemiology of the disease in California was air-borne dissemination of the causal organism. This appeared to be the only explanation for laboratory outbreaks, and a logical explanation for some of the naturally-occurring outbreaks described in the literature.

Since *C. burnetii* is shed in the milk of infected cows (23, 34), sheep (7, 35) and goats (7, 35), and may be present in the placenta of cows (39) and sheep (34),

and also in the feces and nasopharyngeal secretions of sheep (34), ample opportunity is provided for contamination of the environment with the rickettsia, thus facilitating the widespread dissemination of the agent, and eliminating the necessity for close or intimate exposure to the infective source. Many of the human cases of Q fever with no history of direct exposure to livestock might be accounted for by such a mechanism of transmission. Such a premise derives tenability from the fact that *C. burnetii* has been recovered from the dust-laden air of premises harboring known infected dairy cows (13), sheep (13) and goats (34).

PROPHYLAXIS

Vaccines

For many years, the only practical rickettsial vaccine, from the standpoint of both availability in large amounts and ability to confer protection, was that against Rocky Mountain spotted fever. Formerly prepared from infected tick tissues, it is now made from rickettsiae cultivated in the chick embryo; the method of preparation (11) is similar to that originally developed for typhus vaccines.

Epidemic typhus vaccine was developed to its present stage of efficacy during World War II, and used on a mass basis. The results of these extensive trials, which are now well known, leave little doubt that immunization with this material confers practically complete protection against epidemic typhus.

The development of a vaccine against scrub typhus is still essentially in the laboratory stages. Vaccines have been prepared from the spleens or lungs of infected mice, cotton rats, and white rats (6, 15, 60) and from agar tissue cultures of rickettsia (46). Aside from the fact that the manufacture of these vaccines on a large scale by these methods would be difficult (60), their protective value for man has yet to be determined. In one field trial (8), in which a vaccine prepared from cotton-rat lungs (15) was used, the incidence of infection among both vaccinated and control groups was too low to admit of valid conclusions. In another trial (5), a vaccine prepared from rat tissues (60) was tested, and found to be of no value in reducing the morbidity or mortality from scrub typhus. In view of the marked differences in antigenic structure known to occur among strains of *Rickettsia orientalis*, it is not impossible that the failure of the vaccine in the trial just mentioned may have been due to a lack of correspondence between the infecting strains and that employed in the vaccine. (Chloromycetin has also been tested as a prophylactic against scrub typhus,—see below.)

The renewed interest in Q fever has naturally led to studies on specific prophylaxis against this disease. Smadel and his associates (61), using vaccines prepared by the ether-extraction method employed for typhus vaccines (11), found that guinea pigs could be protected against lethal doses of *C. burnetii*, although the immunity was not absolute, as many animals developed non-fatal febrile episodes of short duration. In man, inoculation of the vaccines was followed by the development of complement-fixing antibodies.

Meiklejohn and Lennette (41), investigated the protective effect of similar vaccines in laboratory personnel exposed to infections. Administration of the

vaccine was followed in most individuals by the appearance of complement-fixing antibodies, although the amount of material required to elicit the antibody response varied considerably.

As the antibody level tended to fall considerably within four to five months after immunization, it appeared desirable to follow the complement-fixing antibody titer after the initial course of inoculations (three injections of 1 ml each), and to give single recall injections of vaccine to those persons continually subjected to exposure and whose titers had fallen to 1:8 or less.

Any protection afforded by a vaccine to a group of laboratory people continuously exposed to potential infection is difficult to assess in view of the numerous opportunities for sub-clinical, immunizing infections to occur. During the past two years, an intensive program of Q fever investigations has been carried on, both in the laboratory and field, and nearly 100 persons have been vaccinated. Although not all have been exposed to the same risk of infection, it is nevertheless remarkable that no cases of frank clinical Q fever have occurred amongst our vaccinated personnel; one remembers that Q fever has been notorious for its high morbidity in laboratory workers. The one overt infection which occurred in this laboratory was in an unvaccinated serologist.

Arthropod Repellents and Toxicants

During the past few years, much work has been carried out on the control of arthropod vectors of the rickettsial diseases. It is possible here to cover only the major points in this highly interesting field.

Lice. The louse powder, known as MYL (pyrethrins; N-isobutylundecylenamide; 2,4-dinitroanisole; and Phenol S) was developed before DDT was tested and recommended for use. It is dusted between the skin and the innermost garment, and between all layers of clothing. Application of approximately one ounce destroys all lice present and prevents reinfestation for about one week.

DDT is superior to MYL, as its action is not affected by the degree of humidity and it retains its effectiveness for several weeks. It has generally been applied, as in the case of MYL, as a powder containing DDT in diluents such as pyrophyllites or talcs. It can also be used to impregnate clothing; garments dipped in solvents containing 1 per cent of DDT will retain their louse-killing properties for six months, and through two to four launderings in warm soapy water (32). The value of MYL and of DDT in control work is attested by the rapidity with which an outbreak of typhus in Italy was arrested (67).

Fleas. While DDT is effective against fleas, its action is relatively slow. Consequently, the potentialities of a long list of substances have been investigated, and among the more promising, according to Knipling (33) are the 1,5-pentanediol monoester of caproic acid; (N-(*n*-amyl)imide of 1,2-dicarboxy-3,6-endomethylene-4-cyclohexene; and the tributyl ester of phosphoric acid.

Mites. Dimethyl phthalate and dibutyl phthalate were applied successfully during the last war to the protection of man against the vectors of scrub typhus. Because of certain disadvantages associated with each, their use as a clothing impregnant was replaced by a mixture of equal parts of benzyl benzoate and

dibutyl phthalate in an emulsion (33). Benzene hexachloride (gamma isomer) has been found useful for area control of trombiculid mites (38).

Ticks. Smith and King (66) have recently reported the results of field studies of tick repellents. According to their findings Indalone (1,2-*H*-pyran-6-carboxylic acid, 3,4-dihydro-2,2-dimethyl-4-oxo-butyl ester); hexyl mandelate; dimethyl carbate; ethyl beta-phenylhydracrylate; 2-butyl-2-ethyl-1,3-propanediol; 2-phenoxyethyl isobutyrate; and diethyl phthalate are among the highly effective repellents, and appear to be safe for use either on clothing or by direct application to the skin. Other effective repellents, considered safe for use on clothing but not when applied to the skin, are N-butylacetanilide; 2-[2-(2-ethylhexyloxy)ethoxy] ethanol; Thanite (fenchyl and isobornyl thiocyanoacetates); hendecenoic acid; isobornyl 4-morpholine-acetate; and 2-phenylcyclohexanol.

CHEMOTHERAPY

With the discovery of chloromycetin and aureomycin, there became available for the first time an eminently effective and satisfactory treatment for rickettsial infections.

The first clinical trials with chloromycetin were conducted in Bolivia (43) and in Mexico (59) against epidemic and murine typhus. While the results of these preliminary trials were highly encouraging, conclusive proof of the value of this drug in rickettsial diseases was obtained by Smadel and his collaborators in the treatment of scrub typhus in Malaya (64, 65). These investigators have treated more than 100 cases of scrub typhus, among which there were no fatalities, although many of the patients were seriously ill when therapy was initiated. When the studies were first begun, large doses of the antibiotic were employed but it has since been found that an initial oral dose of 3.0 g followed by 0.25 g every three hours during the succeeding 24 hours constitutes an adequate regimen (57).

The usefulness of chloromycetin as a prophylactic has also been investigated (58, 63, 62). In the first field trial, 46 volunteers were exposed to infection in hyperendemic areas of scrub typhus. Twenty-two of the volunteers received 1.0 g of chloromycetin daily in divided doses throughout the nine day exposure period and for 13 days thereafter; the 24 volunteers in the control group received equivalent dosages of calcium lactate for 22 days. Seventeen of the 24 volunteers in the control group developed scrub typhus 12 to 21 days after the initial exposure, and were given specific therapy. All of the 22 volunteers in the test group remained well during the first 12 days after the initial exposure, but during the ensuing week several showed signs or symptoms that in retrospect were attributed to an incompletely suppressed infection with *R. orientalis*. Between the thirty-first and thirty-sixth days after initial exposure, however, 12 of the volunteers, including those just mentioned, developed overt scrub typhus. Chloromycetin, therefore, suppressed the clinical disease throughout the period of prophylaxis and for about a week thereafter. Additional trials were subsequently undertaken, from which it was found that chemoprophylaxis can be successfully employed to prevent the full evolution of the disease; in many instances, how-

ever, there occurred mild febrile episodes during which the individual had a rickettsemia (58). However, as pointed out by Smadel, although chemoprophylaxis of scrub typhus is feasible, practical considerations limit its usefulness.

Chloromycetin has also been used with good results in the treatment of Rocky Mountain spotted fever (45), and its use in the typhus fevers has been mentioned above.

Following the observations of Wong and Cox (71) that aureomycin is effective in the treatment of rickettsial infections in animals, studies were undertaken in this laboratory to evaluate the usefulness of this antibiotic in the treatment of Q fever in man; the early findings indicated that aureomycin therapy has a favorable effect on the course of the disease (37). More recently, the results in 45 patients treated with aureomycin and 25 patients treated with penicillin have been summarized for comparison (9).

In the penicillin-treated series, the duration of fever after therapy was begun ranged from one to 20 days, with a median of eight days. Three (12 per cent) of the patients in this group became afebrile (less than 99.2 F) in less than three days after penicillin therapy was started; seven (28 per cent) became afebrile in five days or less; and the remainder (60 per cent) continued febrile beyond the fifth day. In general, no marked subjective improvement occurred among the patients receiving penicillin, and the pattern of defervescence differed from that observed in patients treated with aureomycin.

In the 45 patients treated with aureomycin, the duration of fever after therapy was started ranged from one to 31 days, with a median duration of three days. Thirteen (29 per cent) of the patients became afebrile in less than three days, 22 (49 per cent) became afebrile in three days, and 32 (71 per cent) became afebrile in five days or less. The remaining 13 patients did not become afebrile until more than five days after therapy was begun; in nine of the patients in this group, however, there was marked subjective improvement within 48 to 72 hours, in some instances accompanied by a relatively marked fall in fever, and followed by several days of low grade fever before the temperature returned to normal. The four remaining patients showed little or no improvement during the course of therapy.

Considered as a whole, the results indicate that aureomycin is of definite value in the treatment of Q fever. From the data available thus far, it is recommended that large doses, in the neighborhood of 4.0 g or more per 24 hours, be employed.

In the treatment of scrub typhus, aureomycin has produced results similar to those obtained with chloromycetin (58), and has proved effective in the treatment of Rocky Mountain spotted fever (20, 51), the typhus fevers (52, 53), and rickettsialpox (50).

It is of interest that both chloromycetin and aureomycin are rickettsiostatic rather than rickettsiocidal in action; Smadel *et al.* (58, 62) have noted the presence of rickettsemia, without symptoms, in cases of scrub typhus receiving chloromycetin, and we (9) have on a number of occasions isolated *C. burnetii* from the blood during the first three days of therapy with aureomycin, and in one instance as late as eight days after therapy had been discontinued and the

patient was clinically well. Treatment of patients with relapses of scrub typhus or of Q fever has indicated that the causal rickettsiae do not acquire resistance to chloromycetin (58, 62) or aureomycin (37).

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