PROCEEDINGS OF THE
Conference on Progress in the
Understanding of Anthrax

Beaumont House, FASEB, Bethesda, Maryland
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Chairman
WALTER J. NUNGESTER
Introduction

The Conference on Anthrax was organized for the Army Research Office by Dr. Wendell H. Griffith, Life Sciences Research Office, Federation of American Societies for Experimental Biology. Dr. Ralph E. Lincoln of the Army Biological Laboratories played an important role in initiating the Conference.

After Dr. Lamanna's thoughtful introductory statements, three papers present material on the occurrence and epidemiology of the disease followed by two dealing with the bacteriology of the causative organism. The pathology of the disease is then presented. Major attention during this Conference was given to soluble toxins and antigens of Bacillus anthracis. Nine papers and many lively discussions were directed to this area with all of its broad implications to infectious diseases in general.

Dr. Lincoln presents with colleagues a great deal of quantitative data dealing with the relative resistance of animals to B. anthracis and its toxins, bacteria in the blood and tissues at various stages of the disease and the relation of such data to the overall picture of the disease anthrax.

The next to the last paper in this compilation deals with anthrax in man and the treatment of patients with this disease. We were indeed fortunate to have an outstanding expert, Dr. Herman Gold, with many years of experience in treating this disease in man, available to present his experiences and to participate in the discussions.

Dr. L. Joe Berry prepared summarizing comments on the Conference. These comments are followed by a summary of problems needing future attention and prepared from participants' notes submitted at the final session of the conference.

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The anthrax question

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Anthrax belongs to the antiquity of bacteriology and immunology. It was the first disease in which a definitive demonstration was possible of the etiological role of a bacterial species in a disease. It was the disease together with chicken cholera which provided the vehicle for the discovery that cultures of infectious agents could be forced to a state of reduced virulence and then employed for inducing specific active immunity. Yet, in spite of the long historical interest in anthrax, understanding of the pathogenesis of the disease is deficient. A continuing challenge exists in understanding the cause of death in anthrax. Nor can it be said that means are completely adequate for specific prophylactic control and treatment of the disease. Limitations of knowledge and methods for prevention and control of the disease are particularly acute for the respiratory type of anthrax.

The causative agent of anthrax, *Bacillus anthracis*, is the only aerobic spore-forming bacterial species able to cause epidemic disease in man and other mammals. The identity of *B. anthracis* as a species distinct from other spore-forming bacteria, particularly *B. cereus*, has been questioned (8). *Bacillus anthracis* does share some characteristics including identity in some spore and vegetative cell antigens with other *Bacillus* species including *B. cereus* (1, 3–5). Yet, there is no question as to the unique nature of the disease picture it induces (2, 5). Among other interesting facts is that the LD₉₀ with spores and vegetative cells of *B. anthracis* is similar but for *B. cereus* and related organisms considerably larger numbers of spores than vegetative cells are required for a mouse LD₉₀ (5, 7). Because of these observations and because there are ample other characteristics to distinguish *B. anthracis* from *B. cereus* (2, 6), the question of the species rank of *B. anthracis* is settled and has been recognized in the most recent edition of Bergey’s *Manual of Determinative Bacteriology*.

The fact that *B. anthracis* is a spore former means that employment of vaccines for inducing immunity to anthrax can proceed along several separate paths. Because transmission of the disease is probably most often by the spread of spores, it is useful to think of producing spore vaccines. Hopefully these vaccines will stimulate mechanisms for specifically eliminating spores entering into the body and before the spores can germinate. To cause infection the spores must germinate, and it is really the vegetative organisms which by their reproduction are the villains in anthrax. The fact is that the spores and vegetative stages of the anthrax organism are antigenically distinguishable though they do possess some antigens in common (3). Thus a spore vaccine cannot be expected to be as effective against the vegetative organisms as would be a vegetative cell vaccine. The antigenic difference between spores and vegetative cells may explain why living and not dead spore vaccines are the better immunizing agents. The living spore vaccine can yield vegetative bacilli by germination and result in better antibody against vegetative organisms than dead spores can. Most certainly vegetative organisms vaccine cannot stimulate production of antibodies against specific spore antigens and thus reduce the possibilities for effective phagocytosis of spores. Consequently, separate roles can exist for spore and vegetative cell vaccines. The final possibility is to produce toxoids for stimulating the appearance of antitoxin. Both intuition and mounting experimental evidence point to an essential role for toxin or toxins in causing the pathology of anthrax infection. Clearly, there is in the study of immunization against anthrax the possibility of focusing effort along these three separate directions of production of antibody against spores, vegetative organisms, and toxins. It is a sad thing that investigators have not had this kind of vision. Since Pasteur introduced an attenuated living spore vaccine students of anthrax immunity have tended to be single-minded and sponsors of a single source of antigen for their attack on the problem of inducing active immunity. What is needed is some serious thought and effort devoted to the composite approach which would aim at producing a vaccine mixture of antigens inducing concurrent resistance against spores, vegetative bacilli, and toxins. This is a call for a three front rather than a single front immunological attack against anthrax.

Before the best of all possible worlds can be achieved, that is, the eradication of anthrax, we can expect some failures against even the best preventive devices invented. Thus we are fortunate an expanding armamentarium of chemotherapeutic agents does promise improved clinical

1 In conducting the research reported in this Conference, the investigators adhered to “Guide for Laboratory Animal Facilities and Care” established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources NAS-NRC.
treatment to meet this threat. Yet, here too, a bottleneck remains unremoved, namely, the means to diagnose anthrax rapidly, particularly respiratory anthrax, in order to decrease the time between exposure and chemotherapeutic treatment. For the therapeutic task it will also be desirable to press forward to gain the knowledge needed to establish the role of toxins so that one can judge the efficacy of reverting to a combined antiserum-chemotherapeutic clinical treatment rationale.

The place of B. anthracis in nature is another aspect of anthrax that deserves continued scrutiny for both its scientific interest and practical significance. Whether or not the anthrax organism is a strict pathogen in the sense that its continued existence as a species depends on the opportunity for infecting susceptible animals remains to be settled definitively. Since man is an incidental and not the primary host, one could hope to eradicate the disease by a ruthless campaign to exterminate infected animals if the anthrax organism is a true obligate parasite. Such an approach cannot ignore the possibilities for independent existence of B. anthracis in soil. There is no question that anthrax spores can persist indefinitely in soil. What is not clear is the ability of the organism to multiply in soil as an active part of the soil flora. Such a role would entail an ability to occupy an ecological niche successfully against the competition of possibly better endowed spore-forming soil bacteria. The presence of anthrax organisms in soil could arise solely from contamination with spores arising from material and carcasses associated with active disease. If this can be shown to be true, treatment of infected soil to destroy spores and quarantine measures would promise to be effective adjunct measures in an eradication campaign.

This Conference is an opportunity to summarize current knowledge and to air one’s own prejudices before a critical audience. By bringing together investigators with different interests we hope to have provided a forum for an exchange of ideas and a development of a meaningful statement of the needs for well-balanced future research on the epidemiology, pathogenesis, prevention, and treatment of anthrax. By asking you to submit a manuscript we hope to have guaranteed a thoughtful analysis of current knowledge that can be made available to a public audience by the mechanism of publication. These introductory remarks have been deliberately provocative for the purpose of helping the Conference to an intellectually lively start.

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Distribution and economic importance of anthrax

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Anthrax has always attracted public interest. Like plague, it seems to have a special dramatic appeal. Very recently, for example, there was an announcement in a prominent position in the London Times, that a bull had died of anthrax. Although there are nearly two outbreaks a day in Great Britain, even a single death is news when the press hears of it.

It is not obvious why anthrax should attract so much attention. The fact that people can be infected and that they sometimes die is undoubtedly one reason for the special interest. However, people die of other bacterial infections—without provoking much excitement. Possibly there still lingers some awareness of the high morbidity and mortality in animals and in man before the large scale use of protective inoculation. For example, in 1923 in South Africa it was estimated that between 30,000 and 60,000 animals died of anthrax.

By the beginning of the last war the practical aspects of control had been coped with. Animals could consistently be protected by active immunization and the disinfecting procedures applied to animal products were obviously sufficient to prevent any but the very occasional human infection. The outbreak of the war wakened interest in new quarters, because of the possibility that the anthrax bacillus might have a military use. Much of the interest centered around the development of a safe vaccine for immunizing man, since there were obvious dangers inherent in the use of the living spore vaccines. Therefore, the earlier work of Bail on the pathogenesis of anthrax and on the immunogenic potential of nonliving derivatives of the bacillus was greatly extended, as was also the immunochemoic work of the brilliant prewar Hungarian school. A continuing thread of interest in taxonomy and in diagnostic methods has always remained evident.

A curious feature of much of the research of the past 25 years is how remote it has been from practical experience. For example, there has been a series of papers discussing methods for recognizing the anthrax bacillus and distinguishing it from other bacilli. It is noteworthy that few of these emanated from laboratories involved in the routine diagnosis of anthrax; which is, in fact, one of the simplest examinations that public health laboratories are called upon to make, and one which offers little difficulty to trained staff. Had academic workers been in closer touch with routine laboratories, less time might have been spent on the comparatively simple problem of recognizing Bacillus anthracis and more on developing much needed noninhibitory selective media. It is gratifying to see that attention is again being given to this important field (3, 4). On the academic level, the basic problems of the phylogenetic relationships of the large-celled bacilli, B. megaterium, B. mycoides, B. cereus, and B. anthracis deserved more attention. Smith, Gordon and Clarke (7) in their excellent monograph emphasized the close relationship existing between these bacilli, and indeed considered that B. anthracis should be classified as a variety of B. cereus. However, on the basis of capsulation, motility, immunochemical reactions, and gross morphology it could just as well be claimed that B. cereus was a variety of B. anthracis, or that both were subtypes of B. megaterium, which perhaps qualifies better for the putative ancestral type, in that it is both motile and capsulated. In practice, the capsulated B. megaterium is more often confused with B. anthracis than is the uncapsulated B. cereus.

The war years saw a very great increase in the tempo of work on the pathogenesis and immunology of anthrax both in the United States and in Great Britain. Much of this—to judge by published work—was aimed at developing a vaccine for the protection of man. This object was indeed achieved and a vaccine now exists which has already played an important role in controlling outbreaks of industrial anthrax (1), and which will undoubtedly be used on an increasing scale to immunize workers at special risk. However, the nonliving vaccine is not potent enough as well as being too costly for the routine immunization of domestic animals.

The spore vaccine in widest use is, for all practical purposes, avirulent for domestic animals. It can be produced in deep culture at the rate of 10⁴ doses/liter and a single inoculation will provide virtually certain protection for about a year; but not, it should be emphasized, for more than a year. The nonliving antigen on the other hand is produced at the rate of 200 doses/liter (6) and two doses elicit a lower grade of immunity than that following a single dose of the living spore vaccine. Consequently, it has become accepted that the protective antigen will only be useful for immunizing man where ordinary economic considerations do not apply and where the risks of using the living spore vaccine are not acceptable.

In fact, it is generally considered that the living spore vaccine is inherently superior for use in animals. It is questionable whether this conclusion is justified. As long ago as 1948, Gladstone (2), using a complex triphasic
system of culture, obtained a sterile anthrax filtrate which, without an adjuvant, was as potent an immunogen in guinea pigs as a good living spore vaccine. I had the opportunity of comparing Gladstone’s filtrate with the living spore vaccine, and the latter with current filtrate vaccines and have no doubt that Gladstone’s filtrate was far more potent than any described subsequently. Admittedly, the harvests were small, and the process complex. However, since 1948 there have been considerable technical advances in the production of various kinds of membranes and antifoams; both limiting factors at the time. It might well be that far higher yields could be obtained nowadays with far simpler apparatus.

It is perhaps unrealistic to expect such development to be carried out by commercial firms, since anthrax is a serious problem mainly in countries which cannot pay for sophisticated development. However, there are factors which could make the search for a more potent filtrate vaccine for veterinary use a sensible project, even for predominantly profit-motivated organizations. For if high concentrations of the protective antigen could be obtained in quantity and combined with a powerful adjuvant, an immunity of far longer duration than that following inoculation of the living vaccine might be achieved. This would dramatically improve the chances of eradicating anthrax. Furthermore, such a nonliving vaccine could more easily be incorporated in combined vaccines which will undoubtedly be much in demand as the cost of labor on the farm increases. A further disadvantage of the living spore vaccine is its susceptibility to some of the antibiotics so freely used in animal feeds. Occasional failures to immunize could be due to the elimination of the vaccine organisms before they have had a chance to multiply (5).

It has been shown (8, 9) that anthrax can be virtually eradicated by state-wide annual inoculations with effective vaccines, because the bacillus does not usually multiply in competition with soil organisms and dies out unless conditions are especially favorable. In this respect anthrax differs profoundly from clostridial diseases such as botulism, tetanus and gas gangrene or enterotoxemia which are caused by organisms that can maintain themselves in the soil indefinitely and against which protection requires vaccination for all time.

Since anthrax can be eradicated by inoculation, there must be substantial reasons why this policy is not adopted more frequently. In Great Britain there are approximately 500 outbreaks comprising 600 animal deaths in a year. If the average value of the animals is estimated at $240 the deaths represent a direct loss of about $144,000. The immediate cost of administrative control could bring the cost to the neighborhood of $250,000. Since virtually all the anthrax in Great Britain stems from the use of imported feeds, complete control would result if all the animals receiving supplementary feed were inoculated annually. The bill for vaccine alone would come to approximately $700,000. If the cost of

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**Fig. 1.** Outbreaks of anthrax per million susceptible stock.
administration and labor is added, eradication by the inoculation of all animals at risk would seem to be an expensive alternative to the current methods of control in which only the animals involved in an outbreak are immunized. Since five of seven human cases occurred following exposure to imported hides or hair, the incidence of human anthrax could not be noticeably affected by the immunization of animals in Britain.

The situation in regions such as the Middle East, East Africa or Latin America is very different. There, where climate favors sporulation and a persistent contamination of the grazing, and where an extensive and semi-nomadic system of stock raising makes close veterinary supervision difficult, large-scale immunization becomes virtually the only means of limiting important losses in livestock, and of reducing the considerable number of cases in man.

A discussion of the economic importance of any disease has to be based on sensible guesses. Everyone who has had practical experience of controlling disease will have been disconcerted by gross deficiencies in the statistics available. The Office International des Épidizooties (OIE) attempts to provide a world-wide consensus of the incidence of animal diseases. The figures given for the incidence of anthrax in different countries are not strictly comparable—some are expressed as new outbreaks and some as deaths—while the efficiency of reporting and diagnosis vary widely from country to country. In 1963, for example, there were 5 confirmed cases of anthrax in stock and 45 in man in Uruguay. In Chile in 1964 there were 849 cases in stock and 222 in man. If these figures are compared with those for Great Britain in 1964, namely 492 outbreaks in stock and only 2 infections resulting in man, the inescapable conclusion is that the reported incidence in stock in Uruguay and in Chile is minute compared with the true incidence.

There is a suggestion that most deaths occur in warm countries with a large population of susceptible animals. However, many such countries also tend to have poor social services and this, rather than climate, might be a reason for the high incidence of anthrax as well as of other infections. A further difficulty results from the arbitrary nature of national subdivisions. Some countries are so large and comprise regions varying so much in social conditions and in climate that regarding these countries as units must give a very distorted picture of the epizootiology and epidemiology of anthrax.

In the industrialized countries of Northern Europe, the temperature is unfavorable for sporulation for much of the year, so that anthrax tends to be self-limiting even though protective inoculation is not widely practiced. The relatively high incidence in Great Britain and in the Low Countries (Belgium and the Netherlands) reflects their reliance on imported feeds. However, despite regular importation, anthrax does not increase significantly and there is an almost negligible spread to man. This is in sharp contrast to the situation in countries such as Chile where the reported incidence in stock is less than twice that in Great Britain, but where there is a 30 times greater frequency in man.

These examples highlight the differences in the problem of controlling anthrax in countries such as Great Britain and in countries where an extensive, pastoral system of stock raising exists. In the latter, large-scale immunization is the sole practical means of control available. It was shown in South Africa many years ago that the examination of blood or organ Smears from every animal that died, or was slaughtered for any reason whatever, revealed about four times as many cases of anthrax as could be detected by examining material only from animals suspected of having died of anthrax. This explained why the immunization of animals in known infected localities could limit the spread of anthrax, but not eradicate it. However, when it was realized how much the true incidence exceeded the known incidence, mass immunization campaigns were initiated which resulted in the eradicating of anthrax from some of the worst affected regions. In 20 years the number of deaths in the whole country fell to 500, a decrease of over 99%. Anthrax can be eradicated from pastoral areas provided that the necessary organization exists and a completely dependable vaccine is available.

The accompanying map (Fig. 1) shows the number of new outbreaks of anthrax per million susceptible animals reported in 1964. No reports are available from certain important regions such as China, and there are serious doubts concerning the low incidence reported from certain other countries. Obviously, there are unavoidable gaps in the data collected and processed by international organizations such as the OIE, FAO and WHO, and some published figures appear to have only a tenuous connection with reality.

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Epidemiology of anthrax in wild animals and the control of anthrax epizootics in the Kruger National Park, South Africa

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Although anthrax has a very wide distribution throughout South Africa, it is most prevalent in certain parts, like Griqualand West, the Transkei and Eastern Cape Province, parts of the Western Transvaal, Natal, and the Transvaal Lowveld (Henning, 1956).

It is associated with the soil, occurring enzootically among herbivorous animals that are pastured on infected veld. In endemic areas where protective inoculation of animals is not possible, extensive epidemics are sometimes experienced. This is particularly true of wildlife sanctuaries such as the Kruger National Park.

The anthrax epizootics, which will be dealt with in this paper, were not the first, nor, to be sure, will they be the last to hit the Kruger National Park.

From old records and Rangers’ diaries describing mysterious fatalities among wild animals (but with symptoms typical of anthrax), it would appear that mortality has probably been caused by anthrax among game animals since earliest times, and fairly extensive epizootics were experienced during 1923-1924, and again possibly during 1941.

Anthrax is therefore not a foreign disease in the Kruger Park, and one could rightfully pose the question: “Why bother with it?”

In the light of the current human population explosion and the clamoring for more land which already augurs a precarious future for game sanctuaries in general, the answer would be that decimation of the dwindling game herds by anthrax epidemics can no longer be tolerated as a “natural” phenomenon or regulatory mechanism.

The majority of South African game parks are, as elsewhere, not ecological units in the true sense of the word, and the stage is rapidly approaching when there will be no sources left for recolonization should any epidemic disease decimate the members of a particular species to a dangerously low level. Moreover, mortality from anthrax is often not density dependent at all, and the extreme susceptibility of such rare species as roan antelope makes it imperative that steps be taken to safeguard their future, should their habitat lie in anthrax endemic areas (such as the northern half of the Kruger Park).

Areas where anthrax is endemic among wild animals may also constitute important reservoirs from which the disease might spread to agricultural or settled areas.

It is for these reasons that the research personnel in the Kruger Park have taken a particular interest in the preventive aspect of the anthrax problem, and why they have been forced, on occasion, to improvise methods out of desperation to combat the fearful mortality experienced among some species of game animals during the epizootics in question.

The first of these hit the Park during October and November of 1959 and affected an area of some 1,600 square miles. During this epidemic 101 animals died, of which positive anthrax diagnoses were made in the case of 66 kudu, 17 waterbuck, 7 buffalo, 6 roan antelope, and 1 each of the following: hippopotamus, cheetah, and civet cat. One vulture was found dead next to a carcase, but a positive diagnosis could not be made in this case.

During the period June to October of 1960, a very severe anthrax epidemic ravaged the greater part of the northern section of the Kruger Park, during which it eventually spread over a total area of almost 3,000 square miles and 1,054 carcasses were found. Of these 771 were kudu, 75 waterbuck, 58 buffalo, 41 roan antelope, 28 nyala, 16 zebra, 13 bushbuck, 10 steenbuck, 7 impala, 5 reedbuck, 5 genets, 5 warthog, 4 leopards, 3 elephant, 2 duiker, 2 grysbuck, 2 honey badgers, and 1 each of the following: bushpig, baboon, cheetah, and lion.

The last epidemic was experienced during the winter of 1962, when a relatively small number of animals (37 kudu and 3 waterbuck) died in an area of only some 150 square miles, which had escaped the onslaught of the previous two epidemics, along the eastern boundary between the Shingwedzi and Letaba rivers.

During this year also 7 hippo died of anthrax in the overgrazed Pafuri area in the remote northeastern corner of the Park, as well as 2 nyala, a jackal, and a civet cat.

Since the summer of 1962 only sporadic cases of anthrax have been encountered (varying from nil to 5 cases per annum), and only in the heavily populated and overgrazed Pafuri area mentioned above. The
species affected were mainly hippo, kudu, nyala, and impala.

Epidemiology

The immediate origin of none of these epidemics could be traced, but there are strong suggestions of some connection with previous outbreaks. The remains of undetected carcasses which were not destroyed during earlier epidemics could possibly have formed foci of subsequent infection of soil and grazing.

The actual trigger mechanism which set off the epizootics is likewise an unknown factor, but may have a climatological link.

During the period of its worst affliction, i.e., June–October 1960, other areas outside the Kruger Park were also hit by anthrax epidemics, some of which caused mortality among wild animals. From May to July of that year, there was a fairly extensive outbreak among game in the southwestern part of the Caprivi strip in South West Africa, during which time 27 kudu, 7 roan antelope, 3 buffalo, 2 reedbuck, and 2 warthog were found dead among others. From the Bechuanaland side of the border reports were received of deaths among giraffe, leopards, and hyenas.

During September some cattle died on farms south of the Crocodile River, which constitutes the southern boundary of the Kruger Park, and during October reports were also received that anthrax was rife among buffalo herds in Portuguese East Africa, some 50 miles from our northeastern border.

The indications in all cases are that, although the outbreaks cannot be directly linked with the amount of moisture in the soil (some of the areas being marshy and others semidesert), they were all associated with marshland, pans, pools in rivers, or other natural waters drying up and with large numbers of animals concentrating at the limited available watering places during the dry season. The particular dry seasons also followed on summer seasons during which particularly heavy rains were experienced and during which time the soil over large areas became waterlogged.

The general impression gained is that of a cyclical nature in the occurrence of anthrax epidemics among game animals and of the probable role of climatic factors as a trigger mechanism. These aspects, however, require considerable additional research and could lead to a stage where one could possibly predict or give warning of an anthrax hazard if a particular set of conditions prevailed.

An analysis of the carcass data in all three epidemics indicates that kudu suffered the most severe losses of all affected species. These animals are undoubtedly highly susceptible and the mortality was generally very high. They were, however, by no means the most common species in the infected area and other, numerically more abundant, species such as buffalo and zebra suffered comparatively very moderate losses.

In terms of relative density of numbers, the most severe losses were experienced among the rare roan antelope and their meager ranks were thinned out in a most disquieting manner. Only five impala died although they are by far the most abundant species in the affected area. All five were in a poor condition and died in a particularly overgrazed area (i.e., Pafuri). This would appear to indicate a most marked difference in species susceptibility, as all animals in the affected zone were equally exposed to the different sources of infection. Both grazing and browsing species were affected.

Some inexplicable manifestations of species susceptibility were also observed. Although the closely related species such as kudu, nyala, and bushbuck are all highly susceptible, their other close relative, the eland, was not affected, and not a single animal of this species died. As pointed out above, roan antelope suffered heavy mortality, but of the closely related sable antelope, not a single individual died, although they are three to four times as abundant in the area under consideration.

As could be expected from their habits, waterbuck and reedbuck are both highly susceptible. With the exception of kudu and steenbuck, significantly larger numbers of male animals died than females, although this may be coincidental. It was noteworthy, however, that in such cases where a species was particularly susceptible, adult animals in their prime constituted the majority of carcasses, although undernourished as well as young individuals were no exception. Very few yearling calves or lambs were found, and it appears that even in the case of kudu, calves less than a year old rarely succumb to the disease.

Only eland, sable, tsessebe, and blue wildebeest of the larger cloven-hoofed species escaped the scourge entirely. This does not necessarily imply complete immunity, however, as black wildebeest, blesbuck, and red hartebeest, which are closely related to the last two mentioned, are known to have died of anthrax in the Orange Free State. Different strains of the bacterium may affect them equally detrimentally during future epidemics.

Hippo and elephant fatalities were experienced, but were relatively few in number, and this may be due to the fact that the majority of these animals watered in the perennial rivers during the period under consideration.

Of the carnivora that fed on carcasses of herbivores which had died of anthrax, fatalities were experienced among lion, leopard, cheetah, saddle-backed jackal, civets and genet cats, whereas hyena and wild dog were unaffected.

No dead vultures or other birds of prey were found, although it is evident from the literature that birds of prey have on occasion died after feeding from anthrax carcasses.

Of the smaller mammals and rodents, no carcasses were found, but these may conceivably have been overlooked by the search teams in view of their small size and the possibility that they may have succumbed inside their burrows or warrens.

The carcass of only one baboon was found, and a positive diagnosis could not be made as putrefaction had
already set in, and the particular team that found it burned it on the spot.

Of the large teams of workmen that were eventually employed for the purpose of tracking down and burning carcases of animals which died from anthrax, none contracted the disease, although they were rather definitely exposed to the risk, despite all warnings and precautions taken. On the other hand, two Portuguese Africans out of a whole village who handled and partook of the meat of some kudu which they found dead in the bush, contracted anthrax and eventually died in the Mission Hospital at Elim in the Republic. The medical officer in charge diagnosed the intestinal form of anthrax in one of the two cases. The other case had contracted the cutaneous form.

**Symptoms of B. anthracis infection manifested in game animals**

Postmortem examinations were conducted only on the earliest two waterbuck cases and on a kudu cow that died during the first epidemic. After it became evident that the animals had died of anthrax, all subsequent diagnoses were made after examination of cutaneous blood smears. Diagnoses made locally were subsequently confirmed by expert opinion from Onderstepoort Veterinary Research Centre.

The animals, on which postmortem examinations were done, were all in excellent physical condition generally. There was a marked subcutaneous edema and congestion of most of the internal organs. The liver, gall bladder, and lymph nodes were enlarged. The spleen was markedly swollen and dark black in color. There was a massive hemorrhagic edema of the lungs and they were practically filled with blood. A severe hemorrhagic pericarditis was also evident.

Externally, on the other hand, the symptoms in practically all the fresh cases found were alike:

There was a flow of blood from the nose and anus, and in the case of buffalo, often also blood-stained feces. The blood was dark red in color and the clotting time greatly prolonged. Droplets of blood-stained serum oozed through the skin especially on the ears (most evident in the case of kudu), the neck, forelegs, and groin. The carcases soon became bloated and putrefaction set in rapidly.

There was not much evidence of subcutaneous or perineal swelling or edema in the zebra carcases that were found.

Animals were rarely observed in the symptomatic stage of the disease, but there is some evidence that death occurred rapidly and suddenly in many instances. The majority of carcases were found in the close vicinity of what were later proved to be infected waterholes.

A zebra was found dead and still warm with a mouthful of green grass on which it had been feeding.

An elephant cow with a suckling calf died in her tracks as she negotiated the steep bank of the Shingwedzi River, after having watered. Death in this case must have been almost instantaneous, as it was noted that the cow fell on her brisket and pinned down the calf trotting in front of her with her head. The calf was unable to struggle free from the dead weight of its mother’s head and died of exposure in this position.

A cheetah was also found which had died while walking along a game path and was found only about 400 yards from where it had drank at a waterhole. It had, prior to dying, vomited an amount of fresh meat from an anthrax carcase on which it had fed.

Later, a case was observed of a kudu bull which had dropped in its tracks and apparently been unable to rise. Another 30 min elapsed however before it succumbed. It lay in the same spot, kicking spasmodically. Breathing was labored and uneven, and there was a frothy hemorrhage from the nostrils. The eyes were bulging and the animal appeared to be suffocating.

In another case a buffalo bull struggled and bellowed for hours during the night in the river-bed below Shingwedzi camp before it died.

A lioness was found “in extremis” near a windmill and was destroyed. It was too weak to move and the trachea was all but closed by a massive swelling around the throat.

From the available data, it would appear that the period elapsing between the contracting of the infection and death varied a great deal in the same and among different species. This apparently varied from 48 hr to 14 days or longer, although it was very difficult to obtain reliable data of this nature in the field.

**Dissemination and sources of infection**

In all our anthrax epizootics the infection was apparently disseminated chiefly by vultures from dead animals on which they fed to watering places which they visited, immediately after gorging themselves, in order to bathe or drink. In this manner a large number of natural waterholes, as well as drinking holes situated at windmills and artificial dams, were contaminated by the birds as they washed off the gore adhering to their feathers, and often also by vomiting excess ingested meat into the water.

Herb animals such as buffalo, and then also elephant, warthog, and other animals that are fond of wallowing in mud, could presumably carry infected mud from one waterhole to the next, and were probably instrumental in the proved contamination of at least one isolated pan. This pan was picketed by game scouts with the express purpose of preventing vultures from bathing there.

The excreta of vultures and all carrion feeders (even rats) have been shown to contain anthrax spores which pass untouched through their digestive tracts and this was probably another important means of contaminating water and grazing in the affected area.

A number of animals died in or around certain waterholes, the carcases were opened by crocodiles, terrapins, and the like, and in this manner the water was infected.

Osteopathia does occur among game animals, espe-
cially during the winter months and in overgrazed zones. However, this cannot be regarded as a factor of importance in the dissemination of the epizootics under consideration. Similarly, blood-sucking insects such as *Tabanus*, *Hippobosca*, and *Stomoxys* spp. as well as other factors such as tick birds, which may transmit the infection through direct inoculation, probably played only a minor role.

In certain instances, however, mortality during the second epidemic, particularly, could not be related to infected drinking water, and here the Pafuri area serves as an excellent example. After extensive patrols on foot and subsequently from the air, all possible infected waterholes were tracked down and made completely inaccessible to game by 6 August 1960. In spite of these measures, animals which now watered in the large perennial river at hand were still dying in substantial numbers until September 5, and other sources of infection were suspected. It was subsequently revealed that carcasses lying about undetected in the veld for a few days were covered with blowflies. These insects scattered at any sign of disturbance and perched in their thousands on leaves of shrubs and trees in the vicinity. In this manner the leaves of a great many fodder plants in the area were possibly polluted with their excreta or carrion particles adhering to their feet.

It is significant that the highest mortality rate at Pafuri occurred among kudu and nyala—animals which are almost entirely of browsing habit, and had of necessity to feed on the leaves and twigs of shrubs and trees during that time, because of the overgrazed state of the habitat. Noteworthy also was the fact that the mortality here dropped considerably after a number of very severe windstorms denuded the vegetation at Pafuri of most of its leaves.

These observations would then appear to confirm the findings of Sen and Minett (1944) that flies and blowflies must be regarded with vultures as important disseminators of the disease.

The most important source of infection was undoubtedly the drinking water, however, and was probably caused either by spores lying dormant in the bottom sludge and brought to the surface by the churning hooves of animals entering the receding waters, or alternatively by polluted vultures (or other carrion feeders) having bathed in such water in their hundreds.

Water and sludge samples of a large number of suspected dams and waterholes were collected, and although the presence of *Clostridium septicum* in practically all cases considerably hampered the isolation of anthrax spores, bacteriologists at Onderstepoort Veterinary Research Centre managed to confirm anthrax contamination in many cases through the inoculation of guinea pigs.

**Suppressive Measures and Hygiene Campaign**

Suppressive measures applied and all initial efforts in combating the spreading of the epidemic were directed chiefly at *a* the incineration of all carcasses of animals which had died of anthrax, and *b* the elimination of all other potential sources of infection and the provision of clean water.

During the early stages of the campaigns, which were impeded to a certain extent through a lack of manpower and transport as well as intimate knowledge of the extent of the area affected, its existing water supplies, etc., the tracking down and burning of carcasses was a very tedious procedure. Scouts had to rely almost entirely on vultures to indicate the location of carcasses and then had to track down possible sources of infection (i.e., waterholes) in the vicinity of such carcasses.

At a later stage, a light aircraft was used (a helicopter would be ideal) to help with the finding of new carcasses as well as with the mapping of all existing natural water supplies in the relative areas. This speeded up the incineration campaign considerably.

It was also decided to burn out the grazing of such areas where mortality was severe, to eliminate another possible source of infection, as well as to facilitate the more rapid finding of carcasses. The burns also played a significant role in that the young growth that soon appeared kept the animals in these areas, and in doing so probably prevented to some extent the straying of affected animals into uninfected zones.

The carcasses of all animals up to the size of buffalo were incinerated on pyres of dry wood. Elephant carcasses were either buried by means of a bulldozer or enclosed by a corral of thornbush, allowed to rot away and the remains were then incinerated.

It soon became evident, however, that despite all attempts to destroy carcasses, the epidemics continued without abatement. An ever-increasing number of new areas were infected in view of the fact that it was often extremely difficult to localize a source of infection and there was no effective means of destroying or disinfecting such polluted water.

It was generally admitted that the disinfection of contaminated water or mud was practically impossible except by the addition of large quantities of poisonous or corrosive antiseptics such as mercuric chloride, phenol, or formalin, which would naturally render such water dangerous to game.

The pumping out or draining of infected waterholes was soon abandoned in view of the danger of further dissemination of anthrax spores. The only practical expedient adopted then was to close such infected waters by means of hedges of thornbush so that animals were denied all access to the water. This presented no obvious solution to the problem, however, as the ability of anthrax spores to maintain themselves in a liquid medium, where they would possibly be subjected to some competition and even predation by the natural aquatic microfauna, was to us an unknown factor.

It was eventually proved that the heavy summer rains which followed have a remarkable cleansing action on such infected waterholes, and animals which subsequently drank there have suffered no ill effects to date.

At that time we did not know this, however, and acting
on the advice of a research worker at the Medical Research Centre in Johannesburg, we decided to experiment with some of the nonpoisonous quaternary ammonium compounds which have powerful antiseptic properties.

The first of these to be used was a substance called didecyl(dimethyl) ammonium bromide (Tetramine), and was applied at a concentration of 1 part in 1,000 parts of water to a known infected waterhole, around which animals were dying in considerable numbers. In spite of widespread scepticism, we were gratified to find that mortality in the area stopped abruptly soon after the addition of the fluid antiseptic. On the strength of these encouraging results, a large quantity of the substance was acquired and applied with particular success to a number of infected windmill reservoirs and a few natural waterholes.

At a later stage, a related substance, alkyltolyltrimethyl ammonium chloride (Hyamine), was also utilized successfully. This was cheaper and had the added advantage of being apparently effective in curbing mortality even when diluted down to 1 part in 10,000 parts of water.

A series of experiments was subsequently conducted at Onderstepoort to ascertain the value of these substances in the destruction of anthrax bacilli. Dr. Cameron (personal communication, 1960), who did the work, found that both substances destroyed the vegetative forms rapidly and completely. In the case of spores, however, the results were less conclusive. After 48 hr of exposure to 1/10,000 dilutions of the substances, there was some evidence that their fatal action on injected guinea pigs was retarded to a considerable extent. The experimental animals still succumbed, however.

Be this as it may, the results which we attained by the application of these substances in the field were too consistent to gainsay their value in such operations. At this stage we can only recommend their use until something better comes to light. It must be stressed that the use of these chemicals was not acceded to without some misgiving and caution, in view of their possible harmful effect of game drinking water treated in this manner for a long period. There is a distinct possibility that both these substances would have a deleterious effect on the rumen-flora of ruminants and such animals may thus develop ketosis or other metabolic disturbances. For this reason only water holes that were rapidly drying up, and others where there was a reasonable influx of clean water (e.g., springs and windmill dams) were treated with these substances, and no harmful effects were noticed among game animals watering there.

It was at this stage that the first signs of abatement of the epizootic were noticed, and the exceptional importance of well-organized patrols and constant vigilance of personnel throughout the infected area was stressed more than ever. In many instances it was now possible to track down carcasses prior to their dismemberment by vultures and carrion feeders, and to prevent the resultant dissemination of the infection. A number of waterholes and pans were picketed by game scouts to prevent vultures from bathing there. The breeding sites of vultures in border areas were destroyed in order to eliminate the danger of local infection. The cleaning-up operations were continued incessantly even after the epizootic subsided completely and the whole infected area was systematically combed and rid of all old carcasses and bones. The excreta of vultures at their roosts and around certain pans where they bathed in their hundreds was incinerated by means of gas flame-throwers before the onset of the rainy season.

All windmill dams in the infected areas were provided with concrete floors, and at all windmills which delivered into mudholes, a suitable type of concrete dam, which could be easily disinfected, was constructed. The latter has no overflow into a mudhole and is provided with a ball-valve mechanism and bypass so that water is pumped back into the bore-hole when the dam is filled to capacity. The surplus water does not come in contact with the water in the dam, so there is no possibility of the bore-hole itself becoming infected.

The whole of the infected area was again burnt out at a subsequent stage in order to destroy all possible contaminated grazing, dung residues, and the like.

**Prophylactic Measures**

The suppressive methods related above may also be considered as preventive with regard to future epidemics, but not as permanent security, owing to the infeasibility of eliminating all potential sources of infection.

The only effective and safe prophylactic measure is the building up of an immune stock of game in these areas. Despite the fact that a number of animals in the affected zones must have survived infection and may now be immune, and the inherent immunity of other species is such that they do not succumb to the toxins produced by the particular strain of anthrax in question, their offspring may well be affected during future epidemics.

Although it is possible today to capture a limited number of animals by means of the drug-immobilization technique and inoculate them with one or other of the current avirulent spore vaccines, the method has its limitations.

A concentrated nonliving vaccine would seem to be indicated in this event because in some game animals the use of even a very attenuated live vaccine is not safe, if one judges by the results obtained by Neitz (1936) when he inoculated seven blesbuck with anthrax spore vaccine obtained from an attenuated goat strain and three of their number died suddenly.

The author expresses the hope of nature conservation-minded organizations and individuals throughout the world, that a practical means of mass immunization of wild animals against anthrax may be developed in the not too distant future, for only in this manner will the serious outbreaks which deplete the already meager ranks of game be effectively prevented.
SUMMARY

The onset and course of three anthrax epizootics among game animals in the northern districts of the Kruger National Park, South Africa, as well as the various suppressive measures employed in the combat thereof, are described. A number of interesting manifestations and the symptomatology of the disease are expounded.

Watering points appeared to be the most important source of infection and carnivorous birds, carrion eaters, and flies were instrumental in spreading the disease.

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Influence of altitude in the incidence of anthrax in Peru

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Anthrax disease is very common on the coast of Peru where it is necessary to vaccinate the cattle and goats to avoid the disease. But in the sierra over 2,000 m above sea level (approximately 6,000 feet) where we have 80% of our cattle population, 50% of the goat, and all the sheep and alpaca population, the disease is not present.

In some places of Peru with lower and higher areas such as the case of the Department of Arequipa situated at the south of the country, anthrax is only observed in animals from areas under 2,000 m and so far has not been observed in animals from areas over 2,000 m.

The cause of the absence of anthrax in the livestock kept at over 2,000 m above sea level is not related to the susceptibility of the animals to anthrax because by experimental studies it can be shown that goats and sheep from the sierra are very susceptible to anthrax, and in the case of alpacas (Lama pacos) more than 50% of the inoculated animals die from the disease.

In relation to the cattle from the sierra it was observed in practice that they are highly susceptible to anthrax. It is the custom to fatten cattle brought from the sierra (over 2,000 m above sea level) to Lima situated at 200 m above sea level (about 600 feet). When people neglect to follow the recommendation to vaccinate these animals fattened in Lima many outbreaks of anthrax with heavy losses are observed.

Preliminary studies done at about 4,100 m above sea level (about 13,200 feet) showed lack of the sporulation of Bacillus anthracis when mouse-infected blood was poured into sterile soil; but more studies must be done before high elevation can be credited for the absence of anthrax in high areas of Peru.
Cytology of *Bacillus anthracis*

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The historic discovery of anthrax etiology by Koch in 1877 (14) was also an exemplary exercise in cytology. He scrutinized the life cycle of the bacterium, explicitly described his findings, and recorded his observations with finely drawn figures. His cytology was comparative: a companion study of the hay bacillus was published by Cohn (6) in the same issue, with their two sets of drawings combined in the same plate; Koch also compared the features of anthrax bacilli growing in laboratory culture with those in infected mouse spleen; furthermore, in what may have been the first study of bacteria growing within tissue cells cultured in vitro, he observed the anthrax bacilli after phagocytosis by frog peritoneal epithelium.

This same format seems appropriate to follow here. I want to describe certain phenomena about the cytology and morphogenic cycle of *Bacillus anthracis*, make some comparisons with related bacilli, and show the likenesses and differences that occur when the bacterium is within mammalian cells, illustrating these with both light and electron micrographs. Some new observations will be presented, but mainly I will review past findings. I will not attempt to document generalizations that are made from comparative evidence in other species, which is available in reviews and monographs (4, 21, 31). Nor will I attempt to provide all references on a given point, instead citing only the most recent one if it is inclusive.

Focus on the fine structure of "bacteridium," as anthrax bacilli were first called by Davaine (7), became possible only in recent years, with refinement of sectioning and staining techniques for electron microscopy. There could realistically be only faint hope to discover an unusual identity in the species, or between strains of high and low virulence. Characterization of the fine and ultrastructure of the spores and vegetative cells, however, should provide greater cytological precision to the bacteriology and immunology of the organism. Antiquated terms such as "surface" or "somatic" antigens should become identifiable with specific component structures of the cell. Furthermore, these components become available for isolation and study as much more homogeneous reactants in the parasite-host relationship.

DORMANT SPORES

Free, dormant, resistant spores represent not only the terminus in the life cycle of *B. anthracis* but also are significant in the etiological cycle of anthrax disease. Even though low in the scale of resistance as compared to other bacillary spores, anthrax spores insulate virtual perpetuation of the species in the soil. Their dormancy also seems advantageous for invasiveness in infection via the respiratory route: dormant spores are more virulent than germinated spores or vegetative cells (9) possibly because of persistence in the alveolar macrophages so as to be transported to the lymph nodes and there grow out, rather than to be cleared by the mucociliary stream (22, 28).

When examined unstained by transmitted light, the dormant anthrax spore appears to have three main zones: a central, refractile core; a dark covering; and an enveloping, faintly visible exosporium. Remarkably, all of these were perceived, described, and depicted by Koch (14). They can best be observed, however, in a wet mount by means of a dark-phase contrast microscope. Unless heated, as in one of the usual spore-staining procedures, the core of the dormant spore is not affected by stains; the covering takes up usual basic stains (although they mostly are removed if washed) and avidly retains acid carbol fuchsins; the exosporium is best seen in a wet mount via phase optics and accentuated by use of dilute crystal violet and orange-filtered light (10), or by reaction with specific spore antiserum (33).

Viewed intact in the electron microscope with metal shadowing (Fig. 1), the spore proper (core and covering) usually appears undifferentiated, although the exosporium is plainly visible and detected to have a peripheral fringe. Connecting linkages between adjacent spores have been observed in such electron micrographs (27); I have never been able to confirm such plasmodesms in sectioned preparations and interpret them as artifacts.

The detailed anatomy becomes discernible only after the spores are sectioned and heavy-metal stained, as shown in Fig. 2. The internal anatomy of the sectioned anthrax spore is virtually indistinguishable from that of?

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able water throughout the spore and conceives the chemical ultrastructure to be fabricated as a cross-linked, high-polymer matrix which confers thermostability (2). The exosporium in sections prepared with usual staining (Fig. 2) appears as a thin, undifferentiated, loosely fitting envelope. Because it is the primary physiological barrier and antigenic determinant of the spore, it has been subjected to intensive study in a closely related species, *Bacillus cereus* (10). This study revealed a surprisingly complex physical and chemical ultrastructure. The exosporium membrane apparently is made up of two layers. The one seen with normal staining is actually a basal layer, itself comprised of four sublayers; the macromolecular fabric of these is a hexagonally ordered crystal lattice. A similar ultrastructure is believed to occur in the exosporium of *B. anthracis*. Fragments in sonicated spore suspensions show evidence of hexagonal periodicity (Fig. 4). The second, peripheral layer of exosporium corresponds to the fringe seen in shadowed (Fig. 1) or negatively stained preparations (Fig. 5). When stained with lead hydroxide for extended time, sections of exosporium also disclose the presence of this fringe, made up of hairlike projections (Fig. 6) (10, 11, 28). Chemically this hirsute layer is believed to be a mixed polysaccharide, whereas the basal layer seems to be lipoprotein (16). The exosporium is antigenic and, with the spore coat and cortex, likely contains the antigens specific to the spore. Since the exosporium is outermost on the spore and not penetrated by antibody molecules, it probably is the locus of the “surface” antigens of the intact spore. That is to say, when an anthrax spore enters the blood or other antibody-bearing tissue of a spore-immunized animal, it is the exosporium-specific antibody from among the complex of spore-reacting antibodies that actually reacts with the intact spore. This antigenic surface reactivity has been termed the “specific exosporium reaction” by Tomcik et al. (33).

**Transformation of spores into vegetative cells**

The transformation of a dormant spore into a vegetative cell is now recognized to occur in at least three sequential stages; these have been termed activation, germination, and outgrowth (13). Sometimes the entire transformation is loosely referred to as germination, especially in the older literature. The developmental process is relatable to the physiology of a single spore, but usually occurs at random in a developing population. Synchrony can be induced, however, so that a majority of the population develops in concert. Moreover, washing arrests the population at a given stage of development. These principles have been applied to identify both the stages of transformation of dormant spores into vegetative cells and the stages in sporulation. The ability to obtain synchronization and arrest of a population at a given stage of morphogenesis has enabled deductions about individual cells to be drawn from samples of a population.

The activation stage reversibly conditions the spore to germinate, usually is accomplished by mild heating (e.g., 65°C for 15 min), and results in a changed anatomical
appearance in the electron microscope (Fig. 7); activation, however, does not seem to alter the refractility or general appearance of spores when observed in the light microscope.

The next stage in the transformation is that of germination, which abruptly terminates dormancy and its properties. Activated anthrax spores can be germinated in a solution of adenosine, L-alanine, and L-tyrosine, the requirements for which are rather specific. Germination is accompanied by degradation of a major portion of the spore dry weight, with characteristic excretion of dipicolinic acid, calcium, and peptide into the men-

FIG. 2. Dormant anthrax spore, sectioned and stained with permanganate, uranyl, and lead ions. The main structural components of the spore are labeled: exosporium (EX), spore coat (SC), cortex (CX), core wall (CW), nucleoplasm (N), and cytoplasm with ribosomes (CR). (From Moberly, Shafa and Gerhardt with permission of J. Bacteriol. (17)) X 146,000

FIG. 3. Dormant spore of Bacillus cereus, sectioned and stained as the spore of B. anthracis in Fig. 2. There is little to distinguish the two in fine structure. The arrows point to parasporal inclusion bodies, which are found in both species. (From Gerhardt and Ribi with permission of J. Bacteriol. (10)) X 87,000
struum. This important stage of transformation can readily be detected in the light microscope: examination of a wet mount with phase optics discloses a dramatic change from the brightly refractile dormant spore to a darkened germinated one; the change is also evident in preparations stained simply with methylene blue or by the acid-fast procedure (Fig. 8). This latter method is not so commonly used, but has the additional advantages of distinguishing an intermediate stage in the postgerminative development and providing good differentiation of the bacteria within phagocytic animal cells (22, 28).

I have observed that control examination of spore suspensions is not always undertaken by investigators not specifically concerned with cytology, yet profound differences in antigenicity, infectivity, and other properties exist between dormant and germinated spores. Unless quantitative evidence is presented to the contrary, one should assume the presence of significant “contamina-

\[ \text{FIG. 4. Portion of an anthrax spore, negatively stained with phosphotungstate. In various places there can barely be discerned a hexagonal pattern of holes or pits similar to that found in the exosporium basal layer of } B. \text{ cereus (10) but different from that observed in the vegetative cell wall of } B. \text{ anthracis (cf. Figs. 17, 18 and 19). Fragments believed to represent the latter appear in the background adjacent to the exosporium. (Micrograph by Dinah Abram.) } \times 107,000 \]

tion” by germinated forms in the usual preparation of supposedly dormant spores, as illustrated in Fig. 8.

In the electron as in the light microscope, germinated anthrax spores appear quite different from dormant ones. As illustrated in Figs. 9 and 10, the cortex disappears and the core swells, probably from imbition of water. The core wall and membrane, mesosomal infoldings of the core membrane, and the stranded DNA of the nucleoplasm become more differentiated. The exosporium remains intact, and the lamellar outer part of the spore coat also persists.

The third stage in the transformation process is that of outgrowth, which leads to the emergence and division of a new vegetative cell. Because of extensive biosynthesis, a complex of nutrients is required. In the light microscope, the transition is observable as progressive enlargement and elongation; spores stained by the acid-fast method become blue. In the electron microscope, the spore core wall can be seen thicken into the new vegetative cell wall. The nucleoplasm becomes dispersed. Mesosomes tend to form equatorially and a septum develops centripetally. Vestiges of the exosporium and spore outer coat adhere to the cell but eventually are shed, in a manner quite like that observed in B. cereus. An electron micrograph of a cell representative of this stage of development is shown in Fig. 11. Hachisuka, Kojima and Sato (11) also have published micrographs of stages in the outgrowth of anthrax spores.

These outgrowth stages seem to occur identically after engulfment of a spore into a macrophage, although it is more difficult to attain adequate fixation and staining of both bacterial and animal tissue. Electron micrographs of dormant spores after phagocytosis by lung alveolar macrophages are shown in Figs. 12 and 13. An extensive electron-microscopic study of avirulent and virulent anthrax spores developing within mouse spleen has been described in a doctoral thesis by Roth (23).

**Vegetative Cells**

It is the vegetative period in the life cycle of the bacterium that is mainly associated with anthrax disease. The feature distinguishing the anthrax bacillus from most saprophytic relatives, aside from pathogenicity, is the absence of motility; this is confirmed electron microscopically by the complete absence of flagella or their vestiges. A report of motile strains and the induction of motility by bacteriophage (3) subsequently was disclaimed (5).

Unlike spores, the vegetative cells display marked differences in gross appearance as a consequence of in vivo cultural conditions. Actively dividing cells, grown aerobically on artificial liquid or solid media, are nonencapsulated and adhere in long chains. The individual cells retain a cylindrical shape as a result of rectangulation during cell wall septation (Fig. 14). When stained in usual fashion with crystal violet or methylene blue and examined in a light microscope, the cell wall and septa are unstained and only the protoplasm is evident. This
results in the classical textbook picture, resembling an aerial photo of trains of box cars in a marshalling yard. The fine structure of such anthrax cells has been examined in the electron microscope after thin sectioning and metal staining (17, 32). A representative micrograph, containing a cell both in longitudinal and in transverse section, is shown in Fig. 15.

The cell wall appears to have an irregular surface. Debris from the medium may adhere to the wall, but a discrete capsule is not evident. The thickness of the cell wall (about 40 μm) is comparable to other gram-positive types; in some circumstances, it seems layered (24). If cells are ruptured, empty hulls of cell wall may be found and also isolated in mass, as shown in Figs. 16 and 17. Smaller fragments, often appearing as leaflets, display a beautifully patterned ultrastructure with regularly...
angled outlines (Fig. 18). Their identity with the cell wall fabric is indicated from the common pattern that can be discerned in both (Fig. 17).

Just beneath the cell wall is a typical, thin, trilayer-stained cytoplasmic membrane, which often intrudes into

**Fig. 7.** Anthrax spore after activation by heating, triply stained as in Fig. 2. (From Moberly, Shafa and Gerhardt with permission of *J. Bacteriol.* (17).) × 64,000

**Fig. 8.** Light micrograph of anthrax spores stained by acid-fast procedure. Most of the spores are dormant and appear red-ringed and refractile. Some are germinated, however, and appear uniformly red and not refractile. (From Shafa, Moberly and Gerhardt with permission of *J. Infect. Diseases* (28).)

**Fig. 9.** Germinated spore, representative of the early part of this stage of development. The nuclear material is dispersed and stranded. The core wall and membrane are distinct. The cortex mainly has been lost and its space filled by the swollen core. (Micrograph by Betty Moberly.) × 147,000
the cytoplasm to form lamellar or vesicular mesosomes. Such mesosomes usually become associated with the septation or sporulation processes (Fig. 14). In a comparative study of B. anthracis and B. cereus, Pavlova and Katz (19) have reported that differences exist in the structure of intracytoplasmic membranous systems.

With lead staining and good resolution, the cytoplasm is detected to contain dispersed and clustered ribosomes. During active growth, the nucleoplasm may appear quite dispersed, as shown in Figs. 14 and 15; the lobes are identifiable by the characteristic stranding of DNA molecules. Two types of inclusion bodies are found. Some are spherical or ovoid, electron transparent, and delineated (actually by a membrane); from comparative evidence, these quite definitely are identifiable as granules of poly-$\beta$-hydroxybutyrate. In mature (22-hr) cultures that then were ruptured by sonication, there were observed a number of spherical granules which, with negative staining, displayed a remarkable surface periodicity (Fig. 19). It is believed that these may represent the membrane-covered granules of hydroxybutyrate, the membrane giving the ordered appearance. A second type of inclusion body in sectioned preparations appears irregular in shape, clustered, and vesicular (Fig. 15); from recent evidence in studies on B. cereus (8), it seems likely that these areas represent aggregates of glycogen. Altogether the vegetative cell of B. anthracis, like its spore, appears to have little to distinguish it structurally from that of B. cereus, when both are grown under comparable conditions.

When proliferating in blood or other animal tissue, anthrax cells remain together only as septate doublets or short chains before separating; the determinants of chain formation remain unexplained. The shape of individual...
cells is much more rounded at the ends than when grown in vitro. The internal fine structure does not seem different, however.

The principal cytological distinction of parasitizing anthrax cells (and a factor in their virulence, along with toxins) is the formation of capsules. This apparently is a consequence of the presence of carbon dioxide, and can be duplicated artificially. An enormous "cytopathic

**FIG. 12.** Representative survey electron micrograph of a lung alveolar macrophage and phagocytized dormant anthrax spores. Various stages of the phagocytic process are evident. The dark patches in the spores are artifacts believed to be caused by the fixative. (From Shafa, Moberly and Gerhardt with permission of J. Infect. Diseases (28).) $\times 67,000$

**FIG. 13.** Portion of a lung alveolar macrophage after phagocytizing dormant spores, one of which is outgrown but not yet septate. In this bacterium, the nucleoplasm artifactually appears condensed instead of with the typical stranding; remnants of the exosporium and spore coat remain. (From Shafa, Moberly and Gerhardt with permission of J. Infect. Diseases (28).) $\times 27,200$
area" surrounding virulent anthrax cells within mouse spleen (24) subsequently was identified as capsule (26).

A definitive study and review of work on the fine structure of the capsule of *B. anthracis* recently has been provided by Avakyan et al. (1), in which they employ a variety of microscopic, cytochemical, and cytoimmunologic methods. The capsule appears remarkably substructured (Fig. 20). Around the periphery is a membranelike outline that apparently consists of a peptide fringe and a neutral mucopolysaccharide-protein under-

**Fig. 14.** Exponentially multiplying vegetative cell in process of septation (S). Participation of a septum-oriented mesosome (M) as a continuation of the ingrowing cytoplasmic membrane (CM) is evident. The cell wall (CW) and dispersed patches of nuclear material (N) also are apparent. (From Moberly, Shafer and Gerhardt with permission of J. Bacteriol. (17).) X 53,800

**Fig. 15.** Two cells of *B. anthracis* growing exponentially in a casein-digest medium. The cell at top is seen in transverse section, and the cell at bottom, in longitudinal section. Note the typically dispersed state of the nuclear material, the cytoplasm granulated from ribosomes, the ovoid inclusion granules of poly-β-hydroxybutyrate, the irregular vesicular inclusions that are believed to represent glycogen, the trilayered cytoplasmic membrane which sometimes infolds as mesosomes, and the cell wall. (Micrograph by Betty Moberly.) X 39,000
layer. A middle part of the capsule consists of mixed polysaccharide and protein. A third part consists of two sublayers, both reacting cytochemically as acid mucopolysaccharides. In addition to these three layers, transverse septa strate the inner parts of the capsule. This substructural makeup was observed both in light and electron micrographs. Localization of a complex of antigens within the capsule structure was accomplished by use of enzymes and also of capsular and cell wall-reacting, fluorescein-labeled antibody. Apparently the antigenic specificity of the capsule is determined by glutamyl polypeptide, whereas that of the cell wall results from a polysaccharide component.

The hypothesis exists that cytological differences may exist between virulent and avirulent (or “hypovirulent”) strains of *B. anthracis*. Encapsulation usually is so considered; however, the addition of egg yolk with a relatively avirulent inoculum results in the development of capsules and the induction of increased virulence (20). The capsule appears to function in virulence by inhibiting opsonization and phagocytosis (29). Furthermore, a spectrum of strains ranging from relatively high to low virulence all can cause infection and reach a comparable septicemic population level; all tested strains produce toxins and can cause death of susceptible host animals (15). Virulence in anthrax thus appears essentially to be a quantitative, chemical property. Neither Roth (23) nor others have been able to discern meaningful differences in fine structure between strains in relation to their degree of virulence.

What cytologically have been found relatable to virulence are features of the host tissue, probably manifesting the effects of chemical components of toxin or capsule. A principal and intriguing difference is the absence of the phagocytic vacuole membrane around virulent vegetative bacilli in spleen, even those phagocytized after being autoclaved and whether capsulated or not. The vesicular membrane, however, is present around hypovirulent, nonencapsulated, vegetative bacilli (26), and also around both avirulent and virulent spores (23). Seemingly, virulent vegetative bacilli produce some unknown cytotoxic factor, which is not demonstrably associated with either the capsule or the heat-labile toxin complex.

Several other host cytologic responses relatable to virulence have been reported by Roth (23), including a marked increase in eosinophils and plasma cells, nuclear pyknosis, and general splenic hemorrhagia and enlargement. What is not clear from the comparisons, however, is whether the level of infection attained with use of the hypovirulent rough strain was comparable to that with the virulent smooth one. That is, if compensatingly larger inocula were employed (a factor of 10,000 exists between the two variants), then the host responses to the hypovirulent strain might be like those to the virulent one.

**SPORE MORPHOGENESIS AND SPORANGIAL AUTOLYSIS**

The closing sequence of events in the life cycle of the anthrax bacterium encompasses the formation of the spore and the demise of the sporangial mother cell. The mechanisms that regulate sporulation in genetically competent strains are unknown. Chemical control akin to that of hormones is suggested by the action of “sporogen,” a crystallized factor that seems to trigger sporulation of vegetative cells placed in a minimal medium (30). For the most part, however, the investigator seeking to bring about sporulation is left to art and artfulness. The most important environmental factors seem to be a high degree of aerobiosis and a general depletion of external nutrient; perhaps both factors contribute to the well-known failure of anthrax bacilli to form spores during the course of infection in the animal body, so long as the animal lives. Once a vegetative population has ceased active division and becomes granular (with poly-β-hydroxybutyrate), the cells can be transferred to water and will proceed to sporulate “endotrophically.” This and other evidence indicates that sporogenesis is entirely an endogenous process.

The cellular morphology of sporulation is observable in the light microscope, especially with phase optics. A series of stages has been so recognized in *B. cereus* and correlated with certain cytological and biochemical changes in the population (12). The actively growing
FIG. 17. Enlarged portion of a cell wall hull and fragments nearby, in both of which the same patterned ultrastructure was found. (Micrograph by Dinah Abram.) $\times 112,000$
Fig. 18. Group of fragments from sonicated vegetative cell suspension. The crystal-like leaflets are believed to arise from the cell wall. (Micrograph by Dinah Abram) $\times 234,000$
vegetative cells, undifferentiated and occurring in long chains when in artificial medium, characterize the starting stage. When the cells cease dividing, separate, and become granular, a second stage is reached. In a third stage, the first grossly observable evidence of sporulation occurs when a darkened forespore appears and the granules become restricted to a portion of the cell. Refractility begins to appear in the early transitional stage both in a given spore and in distribution among the population. Later, the endospores become fully delineated and refractile. In the final stage, the sporangial mother cells autolyze and mature spores are freed.

The fine-structure cytology of *B. anthracis* during sporulation reportedly has been described by Takagi and co-workers and Suzuki et al. (32), with benefit of electron microscopy but an authoring techniques for good differentiation of the cytoplasmic membrane and mesosomes. Consequently, it is best here to rely on the more recent studies accomplished with the apparently identical species, *B. cereus*. A definitive review and study have recently been published by Ellar and Lundgren (8).

Sporule morphogenesis actually begins in a mature vegetative cell at the granular stage (above) and proceeds through about six or seven substages. A diagrammatic representation of these is reproduced in Fig. 21. The cytological prelude to sporulation involves mainly a doubling and axial disposition of the nuclear material, and the development of a sporal mesosome near a cell pole. The second substage involves a progressive infolding of the cytoplasmic membrane so as eventually to form a double membrane in the spore septum; accompanying this is the appearance of vesicles in the area behind the developing septum, and a division of the nuclear material so that the spore eventually becomes haploid. The third substage is characterized by closure and rounding of the double membrane so as to delineate a forespore. In the next substage, typical spore structure begins to develop: the nuclear material disperses, cortical material begins to appear between the opposed components of the forespore membrane, and the first fragments of exosporium are deposited. By substage 5, morphogenesis has progressed to a recognizable spore: ribosomal aggregates become evident with lead staining; the nuclear regions become electron transparent; the inner membrane of the initial spore septum has become the core wall; the cortex becomes thickened and layered; the outer membrane of the initial spore septum becomes the layered outer coat; and the exosporium now has developed. The sixth substage described by Ellar and Lundgren involves maturation of the dormant spore and the start of sporangial cell autolysis. To this, I would add a seventh substage, during which autolysis is completed and the dormant spore is freed.

Thus is the life cycle of the anthrax bacterium believed to be completed. Its history is not an unusual one. The species would be obscure were it not for the rare circumstance in which its spores are deposited at the right time, in the right place, and on the right animal. Only then does its adaptive asset of encapsulation enable it to proliferate within the new environment. Its second adap-
STAGE

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Pathology of anthrax infection in animal hosts

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While stationed at Fort Detrick, Maryland, several years ago with the U. S. Army Medical Unit, I had the opportunity to study experimental anthrax, respiratory and parenteral, in susceptible and resistant animal hosts. We performed autopsies on animals dying of the unmodified disease, animals sacrificed serially after exposure to study the sequence of pathologic events, and animals in which the course of the disease was modified by antibiotic therapy. Thus we studied the incidence and distribution of the gross lesions observed at autopsy and the histopathology of these lesions. We also had the opportunity to correlate our pathologic findings with clinical observations and bacteriologic studies. In this manner we tried to gain some insight into the pathogenesis of such experimental anthrax. On occasions, such straightforward "butcher knife and coverslip" pathology when correlated with clinical, serologic, and bacteriologic or virologic data can yield a handsome reward in the study of the pathogenesis of a disease. Those who may be familiar with our work with experimental Venezuelan equine encephalomyelitis (VEE) infection in several animal hosts, I am quite sure, will agree that this was the case. The study of the gross and histopathology of experimental anthrax was not as rewarding as it was in the case of VEE, probably because there is no nice sequence of events in the development of the disease in the animal host, nor in the development of the lesions. This is perhaps best illustrated in the case of sheep in which at 28-hr postexposure to a cloud of spores, there may be a bacteremia and one may demonstrate bacilli in the sinuses of lymph nodes, and yet there is no disturbance in the architecture of the same lymph node, nor are there any significant lesions anywhere. At 56 hr, the classical lesions of naturally occurring anthrax, that is hemorrhage and necrosis, are very much evident. Even though our studies of the pathology of the experimental disease did not define the pathogenesis of anthrax, I feel that we did make some interesting and pertinent observations regarding the nature and distribution of the lesions in animal hosts of varying degrees of susceptibility.

Our studies were confined to respiratory or parenteral (generally intradermal) exposure to a phenolated spore suspension of Vollum VIB-189 strain of Bacillus anthracis prepared at the Biological Laboratories at Fort Detrick in 1957. Anthrax toxin (or toxins) was not available to us for pathologic studies during this period of time (1957–1961). I shall try to portray for you the pattern of pathologic changes observed in the monkey (Macaca mulatta), sheep, dog, and pig without overburdening you with excessive morphologic descriptions. In these species we recognize highly susceptible, modestly susceptible, and resistant animal hosts. The sheep and the macaque represent the highly susceptible hosts, the pig the modestly susceptible host, and the dog the resistant animal host.

Our animals were not free of spontaneously occurring diseases that may be encountered in populations in nature that had not been raised as "disease-free or specific pathogen-free" animals. Our monkeys all had lung mites, our sheep had lungworms and caseous lymphadenitis (Corynebacterium pseudotuberculosis), our pigs had lungworms, and the dogs had parasitic lesions common to the species. The only one of these that posed a problem in the interpretation of experimental lesions or in the separation of spontaneous and experimental lesion was the lymphadenopathy in the pig, because of the diffuse fibrosis and eosinophilia of lymph nodes in this host with lungworm infection.

Spontaneously occurring systemic anthrax in man and susceptible animals has been described as fulminating septicemia in which the basic and principal lesions are those of edema, hemorrhage, and necrosis. Any text on pathology, human or veterinary, will state essentially this. After systematic study of the gross and histopathology of our experimentally produced disease in these different animal hosts, we cannot say anything different. These are the cardinal lesions in the monkey and sheep in respiratory or parenterally induced infections. This is not true in the resistant hosts, the pig and the dog. However, we did not succeed in producing a systemic infection or a bacteremia in these hosts by respiratory exposure. We merely succeeded in producing focal lesions in the lungs and the pulmonary and mediastinal lymph nodes, which I shall treat in greater detail later in this presentation.

Terminal pathology in M. Mulatta

Subcutaneous tissues. Massive subcutaneous edema and cellulitis were generally present at the site of inoculation, the inner aspect of the forearm in animals inoculated with varying numbers of spores intradermally. The
edema was gelatinous and blood tinged at autopsy. The extent of the edema was variable, at times involving the whole arm. This extent or degree of edema did not appear to be purely a function of dose, as massive cellulitis involving the whole arm was seen in animals receiving 500 spores as well as animals receiving 40,000 spores. In general, animals receiving a low dose (500 spores) that survived longer than 5 days after inoculation developed massive edema and cellulitis of the whole arm. One animal that received 500 spores and died 3 days after inoculation had only a minimal circumscribed lesion at the site of inoculation. In contrast an animal that received 40,000 spores and died in 4 days had massive edema of the whole arm.

The connective tissues surrounding the corresponding axillary lymph nodes were also edematous and sometimes hemorrhagic. The corresponding axillary lymph nodes themselves were always edematous and hemorrhagic.

Microscopically this lesion of the arm proved to be an extensive cellulitis with much edema, some necrosis, cellular infiltrates consisting of mononuclear, neutrophilic, and eosinophilic cells, hemorrhage, and a large number of bacilli. The edema was the predominant lesion, much more so than the inflammatory cell infiltrate.

Mediastinum. In animals exposed to an aerosol of anthrax spores, the edema of “exposure or inoculation site” was transferred from the arm to the mediastinal tissues. Edema of the mediastinum with some hemorrhage was a frequent finding. This lesion was characterized by a varying degree of widening of the mediastinum. The tissues had an abnormal glistening sheen. Massive hemorrhagic mediastinitis was not observed in these animals with one exception, an animal exposed to a low aerosol dose that died on the 11th postexposure day with massive hemorrhagic mediastinitis and meningitis. Such a hemorrhagic mediastinitis was observed with frequency in animals exposed via the respiratory route in which the infection was then modified by antibiotic therapy which did not spare the animal, but merely prolonged the course of the disease.

Microscopically, the edematous mediastinum contained granular noncellular eosinophilic material, a small amount of hemorrhage, and a heterogeneous population of inflammatory cells, neutrophils, macrophages, and eosinophils.

Lymphatic system. The axillary nodes in the intradermal challenge animals and the intrathoracic nodes (mediastinal, tracheobronchial and hilar) in the respiratory challenge animals were most severely involved. Other lymph nodes were irregularly involved. Grossly, hemorrhage and edema were obvious. Microscopically, hemorrhage, necrosis of lymphatic elements, monocytic and neutrophilic infiltration of varying intensity, necrosis of blood vessel walls, and phagocytosis of nuclear debris, bacilli, and erythrocytes were the principal lesions.

The extent to which the various lymph nodes were involved was markedly variable. At times, only the axillary or thoracic nodes were significantly altered. Other times, the lymphadenopathy was extensive. In six monkeys exposed via the respiratory route the tracheobronchial and hilar lymph nodes were amazingly intact, although septicemia and extensive splenic involvement were present.

The spleen was frequently enlarged grossly, but not always in both groups of animals. Microscopically, the severely involved spleen was depopulated of lymphoid elements, contained much necrotic cellular debris, and masses of bacilli. Such a spleen took on the appearance of a “bag of bacilli.”

Respiratory system. Pulmonary edema and hemorrhages were observed in a large number of the animals in both groups. The “primary anthrax ulcer” of Fraenkel in the trachea or bronchi was not observed in any animals. Lesions of considerable interest in the respiratory challenge animals were subpleural, circumscribed, nodular lesions 1.0–1.5 cm in diameter. They were firm and discrete, but not encapsulated. The subpleural surface of the lesion was either entirely hemorrhagic or with a light center and a dark periphery. The cut surface of the lesion was dark with a light center.

Microscopically, these nodular lesions proved to be dilated bronchioles containing lung mites and an abundance of acellular eosinophilic material. The walls of these bronchioles were lined by a pseudomembranous hyaline structure, which seemed to replace the normal lining epithelium. The normal peribronchiolar histologic elements were completely disrupted by hemorrhage, necrosis of the hypertrophied lymphoid aggregates, massive invasion of bacilli, neutrophils and eosinophils, and some deposits of fibrin. These lesions in essence represented “anthrax-infected lung mite lesions.”

Cardiovascular system. Myocardial and subendocardial hemorrhages were seen in a few animals. The principal lesion in this system, however, was a necrosis of the walls of small blood vessels, in which the integrity of the vessel walls was completely destroyed and the vessels appeared as rings of noncellular, eosinophilic material. This lesion was seen in lymph nodes, in the meninges when they were involved, and in the anthrax-lung mite lesion. This lesion was always associated with large masses of bacilli, necrosis, and hemorrhage.

Central nervous system. A hemorrhagic meningitis was observed in approximately half of the aerosol challenge group of 28 and in one animal in the intradermal challenge group of 10. The hemorrhagic process was confined to the meninges and did not extend into the brain proper. Edema of the brain was a fairly frequent finding.

Microscopically, the meningitis was characterized by hemorrhage, masses of bacilli in and about the blood vessels, a marked infiltration of neutrophils and monocytes, and the necrotizing lesion of small blood vessels already described as a necrotizing vasculitis. Sometimes these vessels were cuffed by bacilli.

Body cavities. Hydrothorax and ascites were irregularly seen in both groups of animals. Retroperitoneal hemorrhages were infrequently seen.

Gastrointestinal tract. An occasional ulcer in the stomach
or intestine containing a mass of bacilli was observed. Masses of bacilli were also seen associated with granulomatous lesions of esophagostomiasis.

Gastrointestinal tract. Lesions were infrequently seen only in the kidney. Casts in the tubules that resembled hemoglobin casts and degenerative changes of tubular epithelium were observed. These lesions were focal and not believed severe enough to cause renal embarrassment. Bacilli were observed in the glomerular tufts.

Liver. Focal areas of hepatocellular degeneration or necrosis were observed in both groups of animals. Large numbers of bacilli were observed in the sinusoids.

Diffuse necrosis of hepatic parenchyma was observed in only one animal. In this case there was an accompanying infiltration of a variety of inflammatory cells. Areas of necrosis were variable in their location in the hepatic lobule.

Endocrine system. Focal hemorrhagic lesions in the adrenal cortex, sometimes accompanied by necrosis, were the only lesions observed. Clusters of bacilli were present in the areas of necrosis.

Bone marrow. Focal areas of necrosis and varying numbers of bacilli were observed.

Thymus. No significant lesions were observed in either group.

In considering the above described changes, the following pertinent observations may be made:

1) Edema, hemorrhage, and necrosis with varying degrees of leukocytic response were the cardinal lesions in both groups of animals. Edema was particularly prominent at the site of inoculation in the intradermal challenge group, or of the mediastinum in the respiratory challenge group. The inflammatory response associated with this edema at these locations was not striking.

2) Necrosis of lymphatic tissues, blood vessel walls, adrenals, and liver was common to both groups of animals.

3) The intrathoracic lymphadenopathy—mediastinitis—meningitis complex in the aerosol challenge group reproduces the Woolsorter's Disease of man described by Greenfield back in 1882 when he described Woolsorter's Disease as a form of anthrax blood poisoning and the "mediastinal cellulitis" in this disease as secondary to the intense lymphadenitis and hemorrhage of the bronchial nodes.

4) The observation that there can be minimal damage to intrathoracic nodes in the respiratory challenge animals, in the presence of bacteremia and marked splenic destruction suggests the possibility of blood-borne dissemination directly from the lungs, especially in the presence of a lesion such as the anthrax-lung mite lesion described in which there was loss of bronchiolar epithelium, in fact, destruction of the bronchiolar wall.

5) The pulmonary lung mite lesion and the infected esophagostomum lesions indicate a predilection of B. anthracis for preexisting lesions.

ANTHRAX INFECTION IN SHEEP

A serial sacrifice study of sheep exposed to an aerosol of anthrax spores revealed a marked absence of any significant lesions until 56 hr after exposure at which time there was a striking massive hemorrhagic mediastinitis and concomitant hemorrhagic lymphadenopathy of the associated lymph nodes. The hemorrhagic process extended into the neck strap muscles. Blood smears at this time were positive. Pulmonary lesions were absent at this time. Some pulmonary edema was observed in animals sacrificed at 28 and 33 hr.

The only other significant gross observation in these animals was the absence of splenomegaly, which is frequently seen in spontaneous anthrax in the domesticated ruminant.

Microscopic examination revealed the presence of bacilli in a hilar lymph node at 28 hr, although the histologic integrity of the node was completely intact. At 56 hr there was virtual dissolution of these lymph nodes with marked necrosis of the lymphoid elements, hemorrhage, and an infiltration of neutrophils and some eosinophils. Bacilli were abundant in these nodes. Virtual lysis of the spleen was seen at 84 hr.

Only one significant pulmonary lesion was observed in this series of animals. At 56 hr, a lesion of a bronchial wall that extended into the adjacent parenchyma was observed. The submucosa of the bronchus was markedly swollen and filled with neutrophils, bacilli, and some fibrin. The overlying mucosa was still intact, although there was beginning disruption of the lining epithelium.

Several interesting singular observations were made in other studies with this host. Bacillus anthracis was recovered from the lungs of a survivor of an unmodified infection 15 days after exposure. The organism was also recovered from a caseous precapillary lymph node, as well as the lungs of an animal that was treated with antibiotics after exposure and sacrificed on day 15. Once again, we see a predilection of the organism for preexisting lesions. Also obvious is the long survival of spores in the lungs.

In the case of two sheep inoculated with a spore suspension subcutaneously and permitted to experience the full course of the natural infection and disease, one developed a massive cortical necrosis of the kidneys, the other a picture compatible with lower nephron nephrosis, with necrosis of tubular epithelium and numerous hemoglobin casts.

In reviewing these observations in the sheep, one is impressed by the absence of any early significant lesions, even in the presence of a bacteremia, as in the case of the 28-hr animal in which there were demonstrable bacilli in one of the intrathoracic lymph nodes, and yet no significant lesions were present. This may be a reflection of the animal's complete inability to cope with the infection.

ANTHRAX LESIONS IN DOG AND PIG

Although bacteremia was never demonstrated in the dog and the pig following a respiratory exposure, B. anthracis was isolated from the lungs of both at varying intervals, except in those animals sacrificed at 30-days postexposure. It was also recovered on numerous occa-
sections from the intrathoracic nodes of the dog, but only once from a tracheobronchial node of a pig.

Discrete hemorrhagic pulmonary lesions were found in the lungs of dogs sacrificed on days 8, 10, and 11. These lesions were dark, firm, and discrete but not encapsulated. Similar lesions were found in the lungs of pigs on days 8 and 10. Microscopically, these lesions in the dog were intensely hemorrhagic, fibrinous, and cellular lesions that completely obliterated the normal pulmonary architecture. The center of the lesion was massive hemorrhage. Surrounding this hemorrhagic core was a zone comprised of dense masses of fibrin which occluded alveoli, bronchioles, and vessels. Intermingled with the fibrin at the periphery and extending further out into the parenchyma was a dense accumulation of neutrophils, plasma cells, monocytes, and large macrophages. The lesion was suggestive of an infarct. Bacilli could not be demonstrated histologically in these lesions in the dog.

The microscopic pulmonary lesions in the hog were very similar to those of the dog with the exception of one animal in which there was marked dilatation of the lymphatics in the pulmonary septae and in the visceral pleura. These lymphatics contained masses of fibrin and some of them contained bacilli which were observed in sections stained with the Brown and Brenn technique.

The cellular response in these lesions in the hog appeared to be more neutrophilic than in the dog.

The intrathoracic nodes of both the dog and the hog were grossly enlarged. Microscopically, the changes in the dog nodes were essentially those of a reactive hyperplasia. The follicles had enlarged germinal centers and were depleted of mature lymphocytes. Large macrophages containing cellular debris were prominent in dilated medullary sinusoids. Bacilli were not observed histologically.

A reactive hyperplasia of the intrathoracic nodes of the hog was also observed. However, evaluation of these nodes in the hog is difficult because of the coincidental lesions of lungworm disease, namely fibrosis and eosinophilia. Significant lesions outside the thoracic cavity were not seen in either the dog or the hog.

The pulmonary lesions of the dog and hog as described are believed to represent the capability of the resistant animal to isolate or "wall off" the invading organism. The masses of fibrin, and the intense cellular response described in these lesions certainly are a formidable defensive mechanism. Such an intense fibrinous and cellular response was not observed in the monkey or the sheep. In these hosts, the fibrinous and cellular responses were mild and diffuse by comparison.

The reactive hyperplasia of the intrathoracic nodes in these hosts is probably secondary to the pulmonary lesion.

Thus we see in the highly susceptible host a mild cellular and fibrinous response to the invading organism and the resulting generalized necrosis and hemorrhage, and a very intense fibrinous and cellular local response to the invading organism in the resistant host.

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Structural model for the lethal components of anthrax toxin based on ultracentrifuge studies

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Three components have been demonstrated in anthrax toxin which presumably is related to pathogenicity (2, 15-19, 21, 25). One component, essential for the edema activity of the toxin, has been called edema factor (EF) by American workers and Factor I by the British. A second component, apparently identical with the immunizing antigen, has been called protective antigen (PA) and Factor II. The third component, essential for the lethal activity, has been called lethal factor (LF) and Factor III. Since confusion has arisen from the different British and American nomenclature for these components, the combined nomenclature suggested by Dr. H. Smith will be used here. After glass filtration of anthrax culture fluids, components EF-I and LF-III are retained on the filter and can be eluted by high pH bicarbonate buffer. Component PA-II is found in the filtrate. The EF-I has edema activity only when combined with PA-II, and LF-III kills rats and mice only when combined with PA-II. Since the yields of EF-I and LF-III retrieved from the filter are generally low and variable, it seemed desirable to study the whole toxin. Wilkie and Ward (28) have been assessing various methods which might yield components in sufficient amounts for physical detection and characterization. The first approach holding promise was that of ultrafiltration (28) in which only the yield of EF-I appeared appreciably lower than theoretical.

The work described here concerns an ultracentrifuge study of the components of whole toxin which are concentrated in the dialysis sac by ultrafiltration and called hereafter UFR. Observations of various batches made while details of the ultrafiltration method were being worked out by A. J. Gaspar (26) showed that four principal boundaries having sedimentation constants of about 28, 58, 14S, and 19S were always visible with schlieren optics in the analytical ultracentrifuge. The relative amounts of the 14S and 19S material varied with concentration of the UFR in such fashion as to suggest they might be aggregates of the slower components. Consequently, UFR’s are now concentrated only about 500-fold on a volume basis and the faster boundaries are barely visible. Components 2S and 5S were always present, generally in roughly equal amounts although their precise ratio cannot be specified (5). A preparation of partially purified PA-II (21) from which all detectable lethal activity had been removed by successive glass filtrations prior to concentration by ammonium sulfate precipitation was found to contain only a 5S boundary. Other preparations still had residual 2S boundaries, now markedly skewed toward the trailing edge. Since the sedimentation constant for lethal activity in UFR, determined with a partition cell (29), was about 5S, it was clear that LF-III sedimented no faster than PA-II and might, therefore, be the 2S material. To settle this question and that of the origin of the two faster components, of which only 14S appeared to have lethal activity, it was decided to separate the components by the sucrose density gradient technique of Martin and Ames (9).

Materials and Methods

For adequate testing of lethal activity in Fischer rats (2), it was necessary to pool samples from the three buckets of the swinging bucket rotor. For this reason, considerable effort was made to standardize gradient manufacture. The method of Martin and Ames (9) was followed very closely, except for the following details: constant volume of the gradients was ensured by weighing the 5 and 20% sucrose solutions introduced into the two chambers of the gradient making device. The stirrer in the mixing chamber was a heavy, stainless steel wire, bent into a helical coil, which produced very rapid, thorough mixing. With this mixing, gradients formed in 10 min were always linear in refractive index. Gradients were made at room temperature and chilled 10 min to 4° C in a prechilled block of a “Temp-Blok” heater. They were used as soon as possible, since when held overnight, the top third of the gradient was erased leaving the sucrose concentration almost constant. Sample layering was critical. If samples sank below the gradient surface momentarily considerable sucrose diffused in, yielding concentrations approaching 5% on occasion, although the interface still appeared sharp. Flow on the surface was ensured by placing the well-dried tip of the 200 μl pipette very close to the gradient surface and slowly driving out the sample by a mechanically driven syringe connected by rubber tubing. Temperature rise during a 15.5-hr run at 38,000 rpm
in the Spinco model L ultracentrifuge was 4 C and seemed quite reproducible although it could not be measured in an actual experiment, since all gradients had to be used. Samples were collected by puncturing the bottom of the tube, as described by Martin and Ames. Eight drops (about 0.2 ml) from each gradient were collected in one tube and the 24 fractions were stored frozen at -20 C.

Analysis in the Spinco model E ultracentrifuge, equipped with ultraviolet absorption optics and a high concentration bromine filter, was done using a valve-type synthetic boundary cell. Refractive index of the gradient fraction was measured and a buffered sucrose solution of 0.006 lower refractive index was used for layering. The runs were made at 4 C to prevent the loss of biological activity. Sedimentation constants were corrected to 20 C and water (22) assuming a partial specific volume of 0.73 for all the components (9). Relative concentrations of the sedimenting components were based on film traces made with the Joyce-Loebl Mark III B microdensitometer, calibrated with crystalline bovine serum albumin (BSA) (Nutritional Biochemicals Corp. lot no. 5776). Diffusion constants were calculated from boundary spreading during centrifugation (14) corrected by the Lamm equation (22). D calculated for BSA agreed with Baldwin’s value (1). Samples retrieved from the centrifuge cell were stored at -20 C until the other assays could be made by members of the Bacteriology Division (28).

RESULTS

Data are given in Table 1 for the ultracentrifugal analysis of a density gradient fractionation of a UFR preparation. Column 1 lists fraction numbers. Columns 3, 4, and 5 give sedimentation constants of visible components and their relative concentrations. For material with the extinction coefficient of bovine serum albumin at 265 m\(\mu\) the value 1 would equal 0.01%. Except where indicated, the sedimenting material appeared to be homogeneous. For the components specified, diffusion constants were obtained and used to calculate the ratios, listed in column 5, of the observed friction coefficients to those for spheres of the same weight (22). The components are quite symmetrical except for 2.0s which may have an axial ratio as great as 1:4. Theoretical sedimentation constants for various aggregates of the principal 4.8s component are listed in column 2 and correspond closely to constants of observed components. Similar data are given in Table 2 for the fractions of a preparation of partially purified PA-II (21). Even numbered aggregates, the 7.6s dimer and the 19s octamer, are present as well as more highly aggregated material. A 1.0s component, not detected in the UFR of Table 1, is also present. Some UFR preparations probably contained it since the 2s boundaries appeared skewed. The 1.0s component appears to be more asymmetric than the 2.0s, with a possible axial ratio of 1:8.

Lethal activity data of Table 1 suggest that most LF-III must have a sedimentation constant close to the 4.8s of PA-II. The wide spread of activity indicates, however, that a new 3.8s component, not previously observed, may carry lethal activity. Lack of activity in fraction 19 containing considerable 3.8s material probably reflects lack of PA-II. Calculations were made for the expected positions of the two components and diffusion spread about their maxima (24). The calculations agree with the concentration data of column 4 and indicate that less than 1% of the 3.8s material should reach fraction 12. Therefore, the 3.8s component probably cannot account for all the lethal activity.

It is possible that the 3.8s component forms even-numbered aggregates as does PA-II. Purified LF-III described by Smith and Stanley (17) appears to have a sedimentation constant of about 15s and could be an octamer. The material had been concentrated by ammonium sulfate precipitation so aggregation was possible. Also, faster and slower boundaries were observed, prior to final purification, in preparations appearing antigendically homogeneous. Presence of dimer,
TABLE 2. Analysis of density gradient fractions of: A) partially purified PA-II, glass filtered to remove LF-III and EF-I; B) LF-III in filter factor, glass filter eluate contains EF-I also

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Data were obtained as described for Table 1, with one exception. For TTD determinations the fractions were diluted 1:10 in a preparation of partially purified PA-II with plate titer of 16 to 32 units.

of the partially purified PA-II (Table 2), had a single major antigen line, related to but not identical with the principal line of the standard partially purified PA-II. Fraction 20 of UFR was not analyzed but fraction 21 also had a line of this type. With this antigenic character, the 2.0s and 1.0s materials are reminiscent of the "C-1" component of the highly purified PA-II preparation of Strange and Thorne (21). Their principal component, C-2, had a sedimentation constant near 4.8s; prior to removal of C-1 on an alumina celite column the mixture had a second, diffuse boundary possibly of 2.0s and 1.0s materials. Their two components were closely similar antigenically both in immunizing action and on Ouchterlony plates. Despite antigenic similarity to PA-II, the 2.0s material appears not to form a lethal mixture with the 3.8s LF-III in UFR fraction 19. Molnar and Altenbern (10) also have described material closely related antigenically to PA-II but inactive with LF-III.

The 2.0s material also appears similar to PA-II chemically. Wilkie and Ward (28) found that the PA-II peak from DEAE-cellulose chromatography of UFR could be freed of minor impurities by rechromatography on ECTEOLA-cellulose. The purified material appeared antigenically homogeneous but still showed a strong split band on disc electrophoresis. Comparison of the 4.8s material from UFR fractions with the 2.0s material showed each had a strong line on disc electrophoresis at the PA-II position, that of 2.0s seeming somewhat anodic to the 4.8s position.

DISCUSSION

Molecular weights calculated, assuming a partial specific volume of 0.73, for the components 4.8s, 3.8s, 2.0s and 1.0s are 51,000, 34,000, 17,000 and 8,500, respectively. This makes it tempting to suggest that the 4.8s, 3.8s and 2.0s components may be hexamers, tetramers, and dimers, respectively, of 1.0s material. The axial ratios estimated for the components also are consistent with such a hypothesis. Close antigenic similarity between PA-II and 2.0s and 1.0s material and chemical similarity of 2.0s material to PA-II indicate the slower pair probably does originate from PA-II. Although enzymatic breakdown (21, 28) may be occurring, such a simple molecular weight relationship for fragments seems rather unlikely. These and other data discussed below seem to indicate that PA-II is dissociating.

To simplify the discussion of the possibility that PA-II is a hexamer which dissociates readily, the following schematic model will be assumed:

1) The 1.0s subunit has a conformation in which binding sites of two types are concentrated on opposite sides and antigenicity is associated with one side only.

2) The exposed, antigenically active, surface of PA-II is such as to favor aggregation by entropic union.

Tanford (29) has pointed out that the popular term "hydrophobic bonding" is a misnomer for this type of interaction (6). "Entropic union" (7) seems more appropriate since the decrease

1 Tanford (29) has pointed out that the popular term “hydrophobic bonding” is a misnomer for this type of interaction (6). "Entropic union" (7) seems more appropriate since the decrease
3) To stabilize the PA-II hexamer, sites on the inner surface of the subunit require a binding agent that readily escapes from the free subunit.

4) Certain material, present in abundance, can strongly bind to the “inner” surface of the free subunit and alter the subunit so as to preclude reassembly into a PA-II hexamer.

5) Reassociation of the altered subunits into trimers can occur via the “outer” surface and, being an entropic union, is favored at higher temperature.

The nature of a major constituent of culture fluids, a pigmented protein referred to here as chromagen, suggested item 4 of the proposed model. Chromagen appears to be a complex between protein and melanin intermediates (28), the latter probably arising from the protocatechuic acid identified by Chao et al. (4) in anthrax culture filtrates. It has an isoelectric pH near 3.8 and strong chelating activity. Strange and Belton’s observation of a large amount of pigmented material with sedimentation constant 3.0s (20), about that expected for a trimer of 1.0s material, suggested item 5 of the model. Most of the chromagen escapes during ultrafiltration but in some instances boundaries were faintly visible on schlieren photographs and sedimented between the 4.8s and 2.0s boundaries. By contrast, partially purified PA-II preparations had strongly absorbing boundaries with sedimentation constants close to 1.0s. Escape of chromagen during ultrafiltration while 2.0s material is retained suggests chromagen must be mainly in the 1.0s form at 4°C as required by item 5. Similarly, PA-II has slight tendency to polymerize at 4°C, as items 2 and 5 require, since aggregates appear only when PA-II concentration is high.

Candidates for the removable binding agent proposed in item 3 of the model might be a basic polypeptide which is present in large quantity in UFR (28) or divalent cations, since calcium is required for PA-II elaboration (12). By sequestering the binding agent as it is released, the highly charged, chelating melanin intermediates should favor the dissociated state of PA-II. Chromagen in UFR gives rise to two principal bands in disc electrophoresis (Wilkie, personal communication). The major part appears in a diffuse band cathodic to PA-II, indicating that most of its charges are neutralized. A small amount appears in a sharp band, the most anodic of the pattern. It seems likely that many of the minor intervening bands are the 1.0s chromagen in different stages of neutralization, since PA-II antigen was detectable all along the pattern. It seems possible that the charges of chromagen must be neutralized before accumulation of PA-II can occur in the culture fluid.

The proposed model was suggested in part by certain similarities between PA-II and tobacco mosaic virus, TMV. The virus is much more stable than PA-II but their dissociation reactions are qualitatively similar. The TMV requires 4–6 M urea for dissociation, while PA-II can be dissociated into antigenically active parts in 2 M urea (Wilkie, personal communication). Dissociation of TMV is markedly hastened by phosphate ion in 6 M urea (3), while phosphate ion alone appears capable of disrupting PA-II (28). The TMV contains firmly bound divalent cations (8), few in number but vital to the structure (3). A similar requirement for multivalent ions seems likely for PA-II. It is particularly interesting to note that the isolated TMV protein monomer associates by entropic union into a stable trimer (7). Similarity to TMV suggests that PA-II also may be a structural protein. If PA-II does arise from some structure of the bacillus, other proteins may arise too. Since LF-III is antigenically unrelated to PA-II, indicating it cannot share a common subunit, its size relationship to PA-II might reflect a packing requirement. Cell permeability may also impose a limit on the size of the particles (11).

Models invoking nonuniform distribution of binding sites have been proposed for a number of proteins including TMV (7, 27). To choose a specific model, the chemical constitution of PA-II would have to be known, and no choice is implied here. Assumption of total separation of two types of binding sites on opposite sides of the subunit (27) was made purely for convenience of discussion and is not necessary for the validity of the scheme. By the same token, some attachment of the melanin intermediates to the exterior of PA-II could well occur (13) and probably accounts in part for the ubiquitous presence of chromagen as a contaminant (28). Contamination by chromagen of PA-II is, of course, called for by the model as proposed, since the 1.0s chromagen should undergo entropic union with PA-II.

**SUMMARY**

Molecular weights of characteristic proteins associated with lethal activity of anthrax toxin form the simple series: 51,000, 34,000, 17,000, and 8,500. Two factors are known to be required for lethal action. One is the immunizing or “protective” antigen, PA-II. The other, unrelated antigenically to PA-II, is arbitrarily called the lethal factor, LF-III. Close antigenic and chemical similarity between a 51,000 and a 17,000 mol wt protein, both apparently immunizing antigens, suggested that PA-II is a dissociable polymer. Close antigenic similarity with a 8,500 mol wt protein suggested further that PA-II might be a hexamer.

A pigmented protein with high negative charge and chelating activity, probably a complex of protein with melanin intermediates, is also a major constituent of culture filtrates. The “chromagen” exists in two sizes: the smaller with molecular weight probably close to 8,500 and the larger an apparent trimer. A schematic model is proposed suggesting that melanin intermediates enhance the rate of PA-II dissociation and bind to the 8,500 mol wt subunit preventing its reassociation into
PA-II. The binding is assumed to be localized to favor reassembly into a trimer with the antigen groups of PA-II masked. The model accounts for many of the known characteristics of anthrax toxin. It is further supported by similarity to current ideas on the structure of tobacco mosaic virus which undergoes reactions similar in a number of respects to those described here. Similarity to the virus also suggests PA-II may be a structural protein of anthrax. If LF-III were another protein from the same structure, its apparent similarity to PA-II in size and organization could arise from packing requirements.

It is hoped the model will serve at least to stimulate further interest in the chemistry of whole toxin. It seems advisable to learn more about chromagen in particular if only to find ways of minimizing its interaction with PA-II.

I am greatly indebted to Thomas Bates who performed the majority of the ultracentrifuge experiments and to Nicholas Pavlovic and Peter M. Nelson for their aid in some of the later experiments. I am also greatly indebted to Ralph G. Kanode, Jr. and Wallace G. Fee of the Bacteriology Division who performed the biological assays. The stimulating collaboration and discussions with M. H. Wilkie and M. K. Ward have been invaluable in the formulation of the hypothesis presented here and they should be accorded full credit if further data substantiate it, but be exonerated if it fails.

REFERENCES

Characterization of anthrax toxin

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The work of Cromartie et al. (4, 5, 18, 19) stimulated renewed interest in the role of the extracellular products of Bacillus anthracis in the pathogenesis of the disease caused by this organism. In 1954 Smith and Keppie (10) reported the presence of a specific toxin in the plasma of guinea pigs dying from anthrax. Intradermal injection of plasma from these animals into other guinea pigs or rabbits caused edematous lesions in the skin; intravenous or intraperitoneal injection into mice and guinea pigs resulted in death. Subsequently, it was demonstrated that material with similar biological activity is produced in vitro by both virulent and avirulent strains of B. anthracis. For detailed information on the development of current knowledge of anthrax toxin and the characterization of its three recognized components, the reader is referred to the work of Smith and co-workers (10-14), Thorne and associates (15, 17) and Beall et al. (1).

Some confusion has arisen from the use of different nomenclature by British and American authors for what appear to be identical materials. In this discussion we shall follow the recent suggestion of Dr. Harry Smith and use a combination of the two. The component which is essential for demonstration of the edema-producing activity of toxin, called edema factor (EF) by American workers and Factor I by the British will be designated EF-I. The second, apparently identical with the immunizing protective antigen, called either PA or Factor II, respectively, will become PA-II. Similarly, the third component, necessary for the lethal effect of the toxin, will be referred to as LF-III.

For definitive studies on the mode of action of the toxin large quantities of concentrated and highly purified material are required. Ideally, whole culture filtrates without prior fractionation should be used as starting material. Littke is known of the physical and chemical properties of the biologically active material as originally produced by the organism or of the relative proportions of the recognized components existing in unaltered toxic filtrates. It is conceivable that the toxin may be a single, loosely bound entity, which is readily fragmented by the glass filtration usually employed in isolation procedures. In addition, the kinetics and mechanisms of toxigenesis remain to be elucidated. Therefore, the methods used for concentration, isolation and purification should avoid procedures known to alter characteristics of labile proteins and give high, quantitative yields of all active components. Both of these qualifications are essential for quantitative evaluation of the efficiency of a procedure in terms of the starting material. Such methods would also make possible basic studies on the kinetics and mechanisms of toxigenesis. A major objective of work on anthrax toxin in this laboratory has been to develop a procedure meeting these requirements.

Of several possible methods available for concentration of large volumes of culture filtrate, a simple ultrafiltration method was chosen. Small diameter dialysis tubing was used as the filtering membrane in a continuous flow system, under negative pressure. Cultures of the relatively avirulent Sterne (Weybridge) strain were grown in the casamino acids medium of Thorne and Belton (16). After a 24-hr incubation period, bacteria were removed by millipore filtration. In the ultrafiltration system used 40-60 liters of filtrate could be concentrated 500- to 1,000-fold in 4-5 days with minimum attention and personnel effort. All detectable toxic and antigenic activity remained in the sac. The ultrafiltrate residue, hereafter designated UFR, was harvested by “backwashing” with tris buffer. Yields of lethal activity, calculated on a volume dilution basis, and mean time to death in the Fischer no. 344 strain of rats, averaged 50-75%. For reasons as yet unknown, recoveries of edema producing and mouse lethal activity have been consistently lower. There is some loss in activity of the crude filtrates held at 4 C for the time period required for the ultrafiltration processing. However, the differential loss in edema-producing activity and mouse lethality could not be accounted for on this basis.

Electrophoresis of the UFR on paper and cellulose acetate indicated that greatest resolution was achieved with the higher ionic strength buffers and higher currents used for peptides rather than conditions optimal for larger proteins. When UFR was incubated at 35 C, the number of protein staining bands increased initially, then decreased, after 4-6 hr, with a concomitant increase in ninhydrin positive bands. On disc electrophoresis at least 16 protein staining bands were observed in fresh UFR preparations. Upon incubation at 35 C an initial increase in number, followed by a loss of protein bands, was also seen on the disc gels. Biological activity rapidly decreased in preparations held at this temperature for more than 6 hr and was entirely lost after 24 hr. All of these changes occurred upon storage at 4 C but much more slowly.
Early in this work, a proteinase, measurable by the Azocoll assay (8), was shown to be concentrated in the UFR and in partially purified protective antigen prepared by the ammonium sulfate fractionation method of Strange and Thorne (15). This enzyme is probably responsible for the rapid degradation of preparations held at 4°C and above.

Immunodiffusion of longitudinal sections of the acrylamide disc columns against antisem prepared by hyperimmunization of a burro with spore vaccine showed that many of the 16 bands shared common antigenic determinants. At least three components formed continuous lines of immune precipitate down the length of the disc pattern. The presence of three or four antigen components identifying throughout the disc pattern was confirmed by reacting transverse sections of the column against burro antisem in identity plates. These findings suggested that at least three components in UFR were in various states of polymerization, were complexing with other substances, or were extensively fragmented.

Initial chromatography studies of UFR employed the DEAE-cellulose-phosphate buffer programs described by Stanley and Smith (14). Recoveries of antigenic and biological activities of UFR chromatographed with this procedure were very low (25-40%) and all fractions contained significant cross-contamination. Under the conditions of these experiments, loss by enzymatic degradation was negligible. It was believed, and later demonstrated, that the PO₄ ion is in some manner detrimental to the UFR proteins. The heterogeneity of UFR proteins observed in column fractions also suggested enzymatic degradation, or protein-protein interaction, or both.

For further work the development of a chromatography system specifically designed for this mixture of proteins appeared essential. It was found that in ammonium acetate the UFR proteins retained their antigenic and biological activities for at least a week at 4°C. Both activities were significantly lost in PO₄ buffers. Since electrophoresis had shown that the mixture contained both strongly basic and strongly acidic proteins, a wide-range elution sequence was required. It was also found that Cellulose collumns gave more uniform results than the Eastman products.

At a ratio of 16 mg protein/g dry weight of cellulose, DEAE-cellulose was equilibrated with 0.02 M ammonium acetate at pH 7.0 and packed in a column of appropriate size to yield a column bed of 1:6 diameter-to-height ratio. Flow rate was adjusted to 1-1.5 ml/min without pressure. The sample of UFR, diluted 1:9 with distilled water, was applied and the column was eluted stepwise with increasing molar concentrations of ammonium acetate through 0.5 M. Volumes of buffer applied and eluate collected varied with the size of sample and column. As a final step, 0.1-0.5 N NaOH was applied to the column to remove a chromagen which will be discussed in detail later. All procedures were performed at 4°C. Protein was measured by the method of Lowry (6) and antigen activity was determined by Ouchterlony's method (9) with the standard burro antisem or the antisera of rabbits immunized with UFR with complete Freund's adjuvant.

Although rat lethality of LF-III is usually tested by recombination with an optimal number of units of partially purified PA-II (determined by titration), this method does not necessarily reflect combining proportions present in UFR or crude filtrates. Therefore, biological activity was measured by reconstitution, instead of recombination.

Figure 1 is a typical chromatogram of 1 ml of a 719 × concentrated UFR. The total volume of column eluate diluted the sample to 4.65 × concentration. Aliquots of 0.1 ml from each tube were combined and 2 ml of the mixture injected into each of three rats. Average time to death was compared with that of animals challenged with the original UFR diluted to 4.65 × concentration and the total recovery from the column was calculated in terms of the starting material. From these columns protein recoveries averaged 100% and rat lethal activity recovery averaged 80-85%.

If it is assumed that each peak is a discrete component, the volume of each peak could be diluted to 4.65 × concentration for testing alone, or any combination of peaks could be tested at the same dilution. Thus, specific rat lethal activity could be located and related quantitatively to the starting material. Frequently, combination of active fractions has had slightly higher activity than the whole column, suggesting an inhibitor which has not been further explored.

In the chromatogram shown in Fig. 1, peak 1 is a basic polypeptide which was weakly antigenic with antisera of rabbits immunized with UFR but did not react with the burro spore antisem. It contained weak EF-I activity when combined with the PA-II peak. Peaks 2, 3, 5, 6, and 8 contained no biological activity related to toxicity and were too weakly antigenic to be identified.
FIG. 2. Chromatography of 2 liters crude culture filtrate on DEAE-cellulose eluted with ammonium acetate buffer gradient, pH 7.0.

Peak 4 contained 90% of the PA-II component and was biologically inactive alone. In reactions with burro antiserum it presented one strong and one weak antigen band. On disc electrophoresis, this fraction showed a strong split band, reminiscent of C-1 and C-2 fractions of Strange and Thorne (15). Anodic to the main band was a weaker band and a chromagen band. Chromatography of peak 4 through ECTEOLA-cellulose with acetate buffers removed the weak band and some chromagen. The PA-II product gave a single antigen band with all antisera and only the strong split band on disc electrophoresis. Further purity studies are not complete. The fraction may still contain other materials not seen in this diluted state.

Peak 7 contained all of the LF-III activity and some PA-II by antigen analysis. It was lethal to rats only when combined with peak 4. Disc electrophoresis showed one major band with three or four minor ones. Bound PA-II could be removed by dilution and passage through the same column system. When this peak was chromatographed on an ECTEOLA-cellulose column, the LF-III product eluted by 0.5 M buffer gave a single antigen band with burro or rabbit antisera and disc electrophoresis of the fraction showed one strong band and a weak chromagen contaminant. Further analysis for purity has not been completed.

The chromatography method described was also applicable to toxic whole crude culture filtrates. The filtrate was diluted 1:6 with distilled water and applied to the column. Fractions were eluted from the column by the same buffer programs and were evaluated by the reconstitution method described.

Figure 2 is a chromatogram of 1 liter of crude filtrate. There were striking similarities to the UFR graph. The basic polypeptide was seen in peak 1; the large peak 5 contained PA-II; and the large peak 9 contained LF-III. But, there were also significant differences: EF-I in good yield was found in the same fraction as PA-II in peak 5 and peak 9 contained only LF-III activity. The PA-II in peak 5 could be further resolved on ECTEOLA-cellulose columns. The nonantigenic, biologically inactive contaminants in peak 9, detected by disc electrophoresis, could also be removed by rechromatography. Protein recoveries from crude filtrates averaged 27 ± 0.6 mg/liter, excluding the chromagen eluted by NaOH. Total biological activity recoveries, calculated as rat lethality, were approximately 85%.

In 5 µliter antigen wells in microimmunoelectrophoresis, at least 1.02 µg PA-II and 0.95 µg LF-III could be detected with the use of the standard burro antiserum. Two milliliters of crude filtrate, which killed rats in 73 min, contained 17.4 µg PA-II and 6.48 µg LF-III, a ratio of about 3 to 1.

The UFR preparative procedure recovered approximately 11 mg protein/liter or 43.4% of the protein in the crude filtrate (excluding chromagen). Chromatography of the biologically inactive dialysis filtrate remaining after UFR harvest yielded 17.7 mg protein/liter (excluding chromagen protein) or 55% which accounted for the protein apparently lost in the ultrafiltration procedure.

A dialysis filtrate chromatogram presented the same outline of peaks as that of the UFR or crude filtrate, except for the complete absence of protein in the PA-II peak area. None of the dialysis filtrate proteins had detectable antigenic or biological activity. This filtrate apparently contains a large proportion of the low molecular weight proteins or peptides synthesized. The relationship of these dialyzable components to those which are nondialyzable but have similar chromatographic characteristics has not yet been explored.

Not shown in Figs. 1 and 2 and extending beyond peaks eluted by 0.5 M acetate buffer, is the peak of the chromagen mentioned earlier. This material could not be eluted by increasing buffer concentrations to 1.0 M or lowering pH to 4 or both, but could be removed with 0.1-0.5 N NaOH. This protein peak had more than twice the area of the others and represented at least 40% of the protein of samples of UFR, crude filtrate, or dialysis filtrate. The substance existed both in a dialyzable form and in a sequence of aggregates up to visible precipitates in UFR or filtrates. The protein complex was nonantigenic and biologically inactive, showed a strong ultraviolet absorption at 260 m, had an isoelectric point at pH 3.8, and dissociated into a free chromophore group and free apoprotein below that pH.

This material seemed to be made up of protein-bound intermediates of melanin formed in culture filtrates, dialysis filtrates, and UFR on standing. Alkaline hydrolysis of a UFR yielded an ether-soluble compound with strong ultraviolet absorption at 260 m which turned pink on oxidation and was identical with the protocatechuic acid first reported by Chao et al. (3). This dihydroquinone is probably the precursor for the melanin intermediates.

The chromagen complex, which is a ubiquitous con-
taminant in column fractions, is in a highly kinetic oxidation-reduction state, is highly acidic, readily polymerizes, and combines strongly with amine groups and cellulose. Its relationship to or effects upon other proteins in the toxic filtrates are as yet unknown. Since the chromagen or its chromophore group was shown to be capable of Ca\(^{2+}\) chelation, this substance may be responsible for the ultraviolet absorbing, chelating activity in EF-I preparations described by Stanley and Smith (14).

The application of the chromatographic procedure to studies of the kinetics of toxigenesis has been initiated only recently; the results are still very preliminary. Single chromatograms of 12-, 16-, and 48-hr culture filtrates have been compared in peak location, yield, and activity to the standard 24-hr filtrate chromatograms. In the chromatogram of the biologically inactive 12-hr culture filtrate, early synthesis of basic protein, PA-II, and chromagen were seen, but no LF-III was detected. The chromatogram of the fully active 16-hr filtrate presented a confusing, but highly suggestive and interesting picture. Basic protein, PA-II, and chromagen had increased in quantity and a large peak in the LF-III area was well developed. However, antigenic activity was found in other areas of the graph and the LF-III protein area was biologically inactive in all combinations with PA-II from the same preparation or others known to be fully active. The chromatogram of the nontoxic 48-hr filtrate showed extensive fragmentation of PA-II with antigenic activity in several peaks.

To continue meaningful studies of kinetics of production, more rapid preparation of larger quantities of the purified fractions is necessary. A preliminary survey of application of the chromatographic procedure to batch preparations has been made. The dry DEAE-cellulose was stirred in the diluted crude culture filtrate overnight. A column packed with this material was eluted with the stepwise sequence of buffers. It was found that the active fractions could be obtained from crude filtrates within 96 hr by this method in yields comparable to those obtained with the standard chromatography procedure. The full capacities of the system are still under investigation.

In summary, an efficient preparative column chromatography procedure was developed which was adaptable to UFR, crude filtrates, or batch collection. Yields of 80–85% of the initial biological activity were obtained in the fractions studied. The PA-II and LF-III fractions prepared by this method appeared to be pure by immunochemical analysis and disc electrophoresis, but they must be subjected to further analyses in order to meet the standard criteria of purity. The EF-I has been recorded only qualitatively in this presentation. A similar quantitative analysis for this substance by chromatography requires a more precise bioassay procedure.

Free chromophore groups, seen in the dialysis filtrate, may be synthesized in excess of the protein moiety of the chromagen component. Since the chromagen and its chromophore are actively aggregating substances, they may act as absorption centers for other proteins and this capability may account for chromagen contamination in chromatographic fractions. The influence of chromagen upon the physicochemical behavior of those proteins is not known.

The presence of considerable amounts of a basic polypeptide in these culture filtrates might contribute to aggregation with the more acidic chromagen or LF-III protein. While this aggregation might contribute to that observed in ultracentrifugal fractions (2), it apparently does not exert a significant effect upon column fractions from DEAE-cellulose. The basic protein is readily dissociated from acidic proteins by the ion-exchange procedure.

In none of the preparations studied has there been evidence to indicate that the components responsible for lethality in the rat exist as a single chemical entity. The preliminary studies on the kinetics of toxigenesis suggest that they are synthesized at different rates in vitro. The dissociation of LF-III activity from the protein eluted by 0.5 M acetate in chromatography of both the nontoxic dialysis filtrate and the fully toxic 16-hr culture filtrates suggests a kinetic extracellular assembly to yield the protein peak with full LF-III activity at 24 hr. This observation makes it tempting to speculate that the protein may serve as an inert carrier for a small, toxic hapten. The work of A. J. Gaspar (unpublished data) and Molnar and Altenbern (7) demonstrated that separate injections of PA-II and LF-III (within 1–4 hr depending upon which was given first) were fully lethal for the rat. There is the suggestion, then, that these two components act sequentially, rather than in combination, in this species. The sum total of all of the above observations provides basis for further speculation and the hypothesis that PA-II activates some mechanism (possibly enzymatic) in the rat which acts to release a toxic hapten from its protein carrier. The requirement for a contribution by the host of some factor essential for full toxic activity is compatible with the observed differences in response to toxin in different species of test animals.

The excellent technical assistance of H. M. Jacoby, R. G. Kanode, Jr. and W. G. Fee is gratefully acknowledged.

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Pigments produced by *Bacillus anthracis*¹

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Production of pigment by *Bacillus anthracis* was first mentioned by Wright, Hedberg, and Slein (16) and by Strange and Belton (10). Broth cultures grown in liquid media for production of protective antigen produced a purplish-red or purple-brown pigment. Although these investigators described some properties of the pigment, their studies were not extensive. We reported (3, 13) excretion of a soluble pink-to-purple pigment by cultures of *B. anthracis* grown in the chemically defined, liquid medium of Thorne et al. (11).

When grown in tightly stoppered flasks containing 0.5% NaHCO₃ in the medium, *B. anthracis* produced pigmented culture filtrate that had a characteristic broad absorption spectrum centering around 490 μm when measured at pH 8.5 (3, 13). The pigment reversibly changed color with variation in pH. Strongly acidic filtrates were colorless; neutral filtrates, purple; and alkaline, red. This pigment was produced by 15 of 20 strains of *B. anthracis*, but not by 5 other species of *Bacillus* (3, 13). Virulent anthrax strains uniformly produced more pigment than avirulent (12, 14).

The iron content of the medium was the most important factor for pigment production by growing cultures. The maximum amount of pigmentation occurred when the concentration of ferrous sulfate was 200 μm/liter (5). Both ferrous and ferric salts were equally effective, but other metallic ions in the same transitional group as iron did not permit formation of pigment. Pigmentation could be detected at 6 hr and increased to a maximum at 48 hr. No pigment formed in cultures grown without addition of iron salts to the medium, but addition of iron to filtrates from these cultures caused formation of pigment. The latter pigment had characteristics identical to the pigment formed when cultures were grown in media containing added iron salts. Thus, some nonpigmented material was produced in cultures lacking added iron salts. This material formed pigment upon addition of the metal to nonpigmented culture filtrates. Both virulent and avirulent strains responded in the same fashion to the addition or deletion of iron salts, but again, virulent strains produced more pigment per unit mass of cellular material (12). In addition, both virulent and avirulent cultures grown in media containing no added iron salts produced another pigment, coproporphyrin III (1).

The difference in pigment production between virulent and avirulent strains prompted further investigations. We speculated that identification of the pigment and definition of conditions required for its production might provide clues to metabolic differences between virulent and avirulent strains. A smooth (virulent) and a rough (avirulent) variant of the Vollum strain of *B. anthracis* were chosen for study (9). Both organisms formed the same pigmented culture filtrate when they were grown under the conditions outlined above (5).

Preliminary studies indicated that pigmented filtrates contained a mixture of colored materials (6). Upon dialysis, some pigment readily passed through the cellulose membrane, but the residuum also remained pigmented. Three pigmented fractions could be separated by chromatography and at least two by extraction with various solvents. One pigment was soluble in water and was not precipitated by (NH₄)₂SO₄; the other pigments were not soluble in water and could be precipitated with (NH₄)₂SO₄. The water-insoluble pigments apparently contained amino acids (6). These substances might be located intracellularly, and rather than being excreted, were released when cells disintegrated. The identity of the water-insoluble pigments has not been established. As described below, the complex of protocatechuic acid with iron has been identified as the water-soluble pigment.

The effect of the iron content of the medium upon formation of pigmented filtrates was similar to the data reported by Garibaldi and Neillands for a pigmented material produced by *Bacillus subtilis* (4). This material also was colorless in the absence of iron. Ito and Neillands (8) isolated the iron-binding substance and identified it as 2,3-dihydroxybenzoylglycine. Following the procedure of Ito and Neillands (8), we isolated from nonpigmented or pigmented anthrax filtrates a watersoluble, crystalline compound that became pigmented upon addition of either ferrous or ferric iron (2). This iron-binding compound was identified as 3,4-dihydroxybenzoic acid, or protocatechuic acid (PCA). The bacterial compound and an authentic sample of PCA had

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identical ultraviolet and infrared spectra, \( R_f \) values on paper chromatography, melting points, and mass spectra (2). In the presence of iron salts, both the bacterial and authentic specimens produced the typical pigmentation when added to culture filtrates of *B. anthracis* from which PCA had been previously extracted.

Labeled PCA is produced when *B. anthracis* is grown in the defined media containing U-\(^{14}C\)-glucose. Nonproliferating, or resting, suspensions of cells also synthesize PCA when glucose is furnished as the only substrate. Aromatic amino acids or phenolic compounds are not effective substrates. In both growing cultures and nonproliferating suspensions, more PCA is produced by virulent than avirulent organisms. Also, more PCA is produced by cultures grown without addition of iron salts to the culture medium than in cultures containing added iron. However, addition of iron salts to nonproliferating suspensions does not influence the amount of PCA produced from glucose. Paradoxically, nonproliferating suspensions harvested from cultures grown in media containing added iron salts synthesize much less PCA from glucose. Perhaps the explanation of this phenomenon is related to the terminal aerobic respiratory pattern of the organism.

Our data indicate that anthrax bacteria when grown under certain conditions form pigments. Although iron salts must be present in the medium for production of pigmented filtrates by growing cultures, cultures growing in medium lacking added iron salts form a colorless material that becomes pigmented upon addition of iron. Coproporphyrin III is excreted only when cultures are grown in media containing no added iron salts; PCA is excreted whether iron salts are added or not. Virulent cultures produce more PCA under all conditions of growth.

The significance of production of PCA by *B. anthracis* is unknown. Quantitative variation in production by virulent and avirulent strains may indicate fundamental metabolic differences. Both PCA and epinephrine have the catechol moiety in their structure, and biosynthesis of PCA may be related to the epinephrinelike activity of anthrax filtrate reported elsewhere at this Conference (15). However, PCA itself does not mimic the action of epinephrine. Our current investigations are attempting to solve these various problems.

REFERENCES

Biochemical and biophysical characterization of anthrax toxin

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The toxin of Bacillus anthracis causes both immunizing responses and pathophysiologic reactions leading to death. Yet, unfortunately, the biochemical and biophysical characterization of the toxin has not kept pace with other areas of anthrax research. We feel that this area is extremely important for the following reasons.

1) First and foremost is the study of the biological action of the toxin at the molecular level; 2) the selection of superior immunizing antigens to be used either singly or in combination; 3) to help provide some basis for selection of strains used for live vaccines or for controlled and variable virulence; 4) for a detailed study of the dynamics of toxin production both in vivo and in vitro; and lastly 5) detailed study of anthrax toxin could greatly enhance man's knowledge in the general fields of bacterial toxins, metabolic products, and pharmacodynamics.

This paper will summarize and compare the published studies on purification of the toxin and its molecular configuration. We hope it will serve as a basis for discussion on what areas of investigation will be most profitable. Since this field may not be familiar either historically or currently and tends to be confusing, we feel that some background and terminology are in order.

BACKGROUND

That sterile filtrates of edematous fluid or of macerated tissue containing the anthrax lesion caused an edematous response or some immunizing effect had been shown as early as 1904 by Bail (2) and was studied more extensively by Cromartie et al. (5). In 1954 Smith and Keppie (13) first demonstrated a toxin which was not cell associated. This toxin, although present in all tissues, was most concentrated in edematous fluid and plasma. It was inactivated by antiserum prepared against avirulent spores. Essentially the identical toxin is produced both in vitro and in vivo (10). We (8, 11) have since shown that toxin occurs in the blood of most animals dying of anthrax, although it is not demonstrable until very late in the course of disease. Different groups have shown that essentially all strains of B. anthracis produce toxin, regardless of their relative virulence (10, 21).

Figure 1 illustrates the nomenclature of the various components and indicates the extent of their biological activity. The English workers first demonstrated that the in vivo produced toxin was composed of two components, and later, from in vitro produced material, identified a third component. They called these components Factors I, II, and III, whereas the American workers have called the same factors edema factor (EF), protective antigen (PA), and lethal factor (LF), respectively. There is general agreement that 1) all three components are serologically distinct, 2) EF and PA synergistically produce edema in guinea pigs and rabbits following subcutaneous injection, and 3) LF and PA synergistically produce the lethal response.

The literature on anthrax toxin and antigen production is limited and confusing. The problem lies in non-uniformity of strain used to produce toxin and in change of the methods of producing, processing, or assaying the products without quantitating the effect of these changes or relating them to a standard. Further confusion results because of the indicia that the molecule exists in various states of aggregation and both biological activity and any other parameters measured vary with the state of aggregation. More will be said about this later.

PURIFICATION

The groups working on toxin purification are using a wide variety of procedures and assay systems, and this situation makes critical evaluation of the experimental data difficult. Table 1 summarizes this problem, and shows that only agar gel diffusion as purity criterion and antigenicity (immunogenicity) assays are common to all four programs.

COMPLETE TOXIN MOLECULE

Most of the research on the effect of anthrax toxin on the host has been done with whole toxin. This whole toxin actually consists of the culture medium after removal of the bacteria by centrifugation and sterilization by filtration through a sintered-glass filter. The characterization of six separate lots of whole toxin according to protein concentration, dry weight, rat lethality, guinea pig edema, and serological activity is presented in Table 2. The high ratio of solids to protein is a reflection of the salts and media components as well as other bacterial products. This whole toxin produces edema, is lethal, and is serologically active.
American nomenclature

English nomenclature

I  (K,Y)  
serologically active

II  
serologically active

III  
serologically active

FIG. 1. Activities associated with toxin components.

TABLE 1. Different methods of toxin production and evaluation

<table>
<thead>
<tr>
<th>Criteria of Evaluation</th>
<th>Research Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>British, Porton</td>
</tr>
<tr>
<td>Bacterial strain</td>
<td>Wright</td>
</tr>
<tr>
<td>Sterne</td>
<td>+</td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>Purity</td>
<td></td>
</tr>
<tr>
<td>Ultracentrifuge</td>
<td>+</td>
</tr>
<tr>
<td>Paper electrophoresis</td>
<td>-</td>
</tr>
<tr>
<td>Agar gel diffusion</td>
<td>+</td>
</tr>
<tr>
<td>Biological properties</td>
<td></td>
</tr>
<tr>
<td>Guinea pig edema</td>
<td></td>
</tr>
<tr>
<td>Mouse lethality</td>
<td></td>
</tr>
<tr>
<td>Rat lethality</td>
<td></td>
</tr>
<tr>
<td>Antigenic activity</td>
<td></td>
</tr>
</tbody>
</table>

Three of the four research groups used sintered-glass filters to separate the three components. Analyses of the individual components are given in Table 3. Although there is some variability among the individual values, the following conclusions appear warranted: 1) EF in combination with PA produces edema in guinea pigs; 2) LF in combination with PA produces rat lethality; 3) none of the components is biologically active individually; and 4) all three components are antigenically active and, although not shown here, distinct.

From this point on, all four groups of investigators used different methods and assays for further purification of the individual components.

EDEMA FACTOR COMPONENT

The various studies on the purification of EF are summarized in Table 4. When guinea pig plasma was ultracentrifuged, the washed deposit was identified as EF. Stanley et al. (16) purified this in vivo produced component about 50-fold with a 20% recovery. However, the EF was still contaminated with 16% normal guinea pig plasma components. Stanley and Smith (17) purified in vitro produced EF 25-fold with 25% recovery. They report that their final preparation was still lethal for mice and was contaminated by at least two other antigens. Based on the observations of Beall, Taylor, and Thorne (3) that EF is selectively adsorbed to sintered-glass filters we (6, 7) have purified this component 197-fold with 38% recovery. The final material produces

TABLE 2. Characteristics of whole toxin

<table>
<thead>
<tr>
<th>Lot</th>
<th>Protein, mg/ml</th>
<th>Dry Weight, mg/ml</th>
<th>Rat Lethality, U/ml</th>
<th>Guinea Pig Edema, liter</th>
<th>Ouchterlony, liter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.24</td>
<td>15.8</td>
<td>32</td>
<td>1150</td>
<td>1:8</td>
</tr>
<tr>
<td>2</td>
<td>1.46</td>
<td>17.4</td>
<td>36</td>
<td>118</td>
<td>1:8</td>
</tr>
<tr>
<td>3</td>
<td>1.07</td>
<td>13.6</td>
<td>37</td>
<td>1116</td>
<td>1:8</td>
</tr>
<tr>
<td>4</td>
<td>1.56</td>
<td>12.9</td>
<td>21</td>
<td>1132</td>
<td>1:8</td>
</tr>
<tr>
<td>5</td>
<td>1.41</td>
<td>14.0</td>
<td>28</td>
<td>114</td>
<td>1:8</td>
</tr>
<tr>
<td>6</td>
<td>1.53</td>
<td>15.0</td>
<td>22</td>
<td>114</td>
<td>1:8</td>
</tr>
</tbody>
</table>

* Culture medium contained 7.5 mg/ml dry weight initially which included 2.2 mg/ml of glucose, 3.5 mg/ml of casamino acids, and 1.6 mg/ml of potassium phosphate.

TABLE 3. Characteristics of toxin components

<table>
<thead>
<tr>
<th>Component</th>
<th>Protein, mg/ml</th>
<th>Dry Weight, Ouchterlony, Guinea Pig Edema, liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF</td>
<td>0.393</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>0.039</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>0.273</td>
<td>1.64</td>
</tr>
<tr>
<td></td>
<td>0.276</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>0.094</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>0.026</td>
<td>1.8</td>
</tr>
<tr>
<td>PA</td>
<td>0.750</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>0.985</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>1.220</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>1.263</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>1.225</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>0.258</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>1.135</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>1.540</td>
<td>11.2</td>
</tr>
</tbody>
</table>

* Dry weight of the carbonate eluting buffer was 14.3 mg/ml in some experiments and 28.5 mg/ml in others. †Shown to be contaminated with LF by Ouchterlony analysis. ‡Dry weight of the initial medium was 7.5 mg/ml.

edema in guinea pigs and only one line of precipitation has been observed on Ouchterlony plates.

PROTECTIVE ANTIGEN COMPONENT

The PA component, because of American and Russian interest in it as an immunizing antigen, has been studied more than the other components. Studies on its purification are summarized in Table 5. Smith et al. (15) found PA in the supernatant of terminal guinea pig serum after ultracentrifugation, and purified it 20-fold with 20% recovery (16). Other procedures used have been 1) alum precipitation (23); 2) trichloroacetic acid-citric acid precipitation (18); 3) ammonium sulfate precipitation (19); 4) sintered-glass filtration (21); and 5) ethanol precipitation (11). All of the above preparations have been
TABLE 4. Purification of edema factor

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Method</th>
<th>Recovery %</th>
<th>Purification, Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stanley et al.</td>
<td>Ammonium sulfate</td>
<td>20</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>DEAE-cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ultracentrifuge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stanley and Smith Fish</td>
<td>Ammonium sulfate</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>DEAE-cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sintered-glass filtration</td>
<td>38</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 5. Purification of protective antigen

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Method</th>
<th>Recovery %</th>
<th>Purification, Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stanley et al.</td>
<td>DEAE-cellulose</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td></td>
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<td></td>
<td>Lyophilization</td>
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<td></td>
</tr>
<tr>
<td>In vitro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strange and Thorne</td>
<td>Ammonium sulfate</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 5.0 precipitation</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Alumina C-gamma gel column</td>
<td>78</td>
<td>156</td>
</tr>
<tr>
<td>Fish</td>
<td>Sintered-glass filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ammonium sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polycrylamide gel column</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

shown to be heterogeneous by Ouchterlony assay or contaminated with the other components or both.

Strange and Thorne (19) purified PA and recovered 25% of the activity but not enough information was given to calculate the fold purification. We (6, 7) have reported 156-fold purification with 78% recovery. This material is not contaminated with extraneous antigens and has retained its serological and immunogenic properties. In some preliminary experiments we used a Diaflo pressure cell and obtained results similar to those using ammonium sulfate. This method is much easier, faster, and more economical for large quantities of material (i.e., vaccine production) than any other method studied so far.

LETHAL FACTOR COMPONENT

The purification of LF is summarized in Table 6. The LF was identified by Smith and Stanley in 1962 (14) and purified 3.5-fold with 24% recovery. Again, following the observations by Beall et al. (3) of selective adsorption on sintered glass we (6, 7) were able to purify this component 1,025-fold with 11% recovery. The resulting product showed only one line when tested by double diffusion in agar. Rat lethality of the final preparation and immunogenicity were not tested.

MOLECULAR CONFIGURATION

During the course of our studies on purification it appeared that the toxin molecule could exist in different forms. What was not clear, though, was whether these forms were aggregates or polymers.

IN VIVO PRODUCED TOXIN

Smith and Gallop (12) found evidence for compounds X and Y in EF (precipitates with ethanol and BaCl₂). Compound Y was postulated to be a converted form of EF, losing its tissue-damaging and virulence-enhancing activity. Later Stanley, Sargeant, and Smith (16) reported that EF had a tendency to aggregate and lose activity and noted that upon ultracentrifugation the peaks depended upon the freshness of the original material and could be altered by very mild treatment. The high molecular weight material could be increased at the expense of the main peak. In 1961, Stanley and Smith (17) called attention to the remarkable chelating action of EF on most metals and speculated on the possibility that the three components might at one time have been joined in a loose complex. When we (6) compared Table 7, the immediate analyses of the serum from six monkeys which had died from anthrax with the analyses of the same sera following 4 day storage at −20 C, the following observations were made: 1) the number of lines on Ouchterlony plates increased without measurable decrease in final titer, indicating dissociation of a complex rather than destruction of a single molecule; and 2) the biological activity, as indicated by edema in guinea pigs and death of rats, was also markedly less after this freezing and storage.

IN VITRO PRODUCED TOXIN

The observation of additional lines of precipitation from PA (Ouchterlony) by Strange and Thorne (19) was interpreted to indicate degradation of this component by a proteolytic enzyme present in the medium. The possibility that this heterogeneity is dissociation of a complex, rather than destruction of a single antigen cannot be discounted. Upon immuno electrophoresis of their “highly purified protective antigen” Wright and Lukas (24) found three immunologically related components differing in electrophoretic mobility. To explain this they have postulated degradation of a basic antigen. The amounts of each compound varied with the age of the culture. Smith and Stanley (14) reported that their final preparation of LF, while being serologically distinct from

TABLE 6. Purification of lethal factor

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Purification Process</th>
<th>Recovery %</th>
<th>Purification, Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smith and Stanley</td>
<td>Sintered-glass filtration</td>
<td>24</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>DEAE-cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hydroxyapatite column</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ammonium sulfate precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td>Sintered-glass filtration</td>
<td>11</td>
<td>1,025</td>
</tr>
<tr>
<td></td>
<td>Sephadex G-25 column</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Calcium phosphate gel adsorption</td>
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<td></td>
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</tbody>
</table>
TABLE 7. Effect of freezing and thawing on in vivo produced anthrax toxin

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Fresh Serum</th>
<th>Frozen and Thawed Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ouchterlony*</td>
<td>G. Pig†</td>
</tr>
<tr>
<td></td>
<td>2 Lines</td>
<td>1 Line</td>
</tr>
<tr>
<td>1</td>
<td>Lost</td>
<td>1:80</td>
</tr>
<tr>
<td>2</td>
<td>&gt;1:32</td>
<td>1:80</td>
</tr>
<tr>
<td>3</td>
<td>1:16</td>
<td>1:20</td>
</tr>
<tr>
<td>4</td>
<td>1:16</td>
<td>1:20</td>
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<tr>
<td>5</td>
<td>1:16</td>
<td>1:20</td>
</tr>
<tr>
<td>6</td>
<td>&gt;1:32</td>
<td>1:80</td>
</tr>
</tbody>
</table>

* Highest titer at which indicated number of lines are visible. † Time to death of two rats following iv injection. § No edema. ‡ Average

TABLE 8. Effect of urea and guanidine acetate on anthrax toxin components

<table>
<thead>
<tr>
<th>Days at 4°C</th>
<th>Components*</th>
<th>Titer by Ouchterlony Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H2O</td>
<td>Guanidine 0.25 M</td>
</tr>
<tr>
<td>Day 0</td>
<td>EF</td>
<td>1:16</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>1:16</td>
</tr>
<tr>
<td></td>
<td>LF</td>
<td>1:4</td>
</tr>
<tr>
<td>Day 17</td>
<td>EF</td>
<td>1:16</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>1:8</td>
</tr>
<tr>
<td></td>
<td>LF</td>
<td>1:4</td>
</tr>
</tbody>
</table>

* Component diluted 1:1 with reagent to indicated concentration. † No line of precipitation formed.

EF and PA, contained two closely associated antigens which had different rates of migration upon agar gel diffusion. We have found, during the course of purification of the toxin components, that the lines formed by LF and EF often show partial identity but are always distinct from those formed by PA. The PA preparations sometimes yield two or three lines which form closer to the antigen well, indicating a greater molecular weight, upon storage of concentrated aqueous solutions or in solutions containing a high concentration of ammonium sulfate. Upon chromatography of either PA or LF on Sephadex G-25 we have occasionally observed the presence of multiple peaks which were still antigenically distinct from the other two major components of toxin. These may represent polymers of different molecular weight.

When the three purified components were incubated with urea and guanidine acetate, as shown in Table 8, EF was stable, PA was destroyed readily by both agents, and LF was more slowly destroyed. These observations indicate that the antigenic activity of PA and LF is sensitive to hydrogen bond disrupting reagents, while that of EF is refractive to these agents.

MOLECULAR WEIGHT

Cromartie et al. (5) associated PA activity with a component which migrated between the beta and gamma globulins on paper electrophoresis. The antigen of Boor and Tresselt (4) was characterized as a gamma globulin-like protein by measurement of precipitation and electrophoretic limits. The possibility of serum components was not excluded. We found that all components of toxin are retained by a Diaflo membrane (Amicon Corp., Cambridge, Mass.) which retains molecules of molecular weight 10,000 or greater. The PA has been shown to have a molecular weight in the order of 100,000 by chromatography on Sephadex G-75 (Fig. 2). The antigenic activity is eluted shortly after gamma globulin and before papain. Since PA migrates through 9% agar, the molecular weight is probably no greater than 100,000. The in vitro produced PA of Strange and Belton (18) had a sedimentation constant of 3 which indicated a molecular weight of 40,000–60,000. J. Gruber and G. G. Wright (personal communication), using the ultracentrifuge, indicated a molecular weight of 80,000 for PA. The molecular weight of LF appears to be of the same order as that of PA. These molecular weight determinations will no doubt have to be reevaluated after we more fully understand the molecular configuration of the toxin.
CONCLUSION

It is obvious by now that the work in this field has presented more questions than it has answered. With the exception of the work on the isolation and purification of the toxin components all the results should be considered to be more of a preliminary nature than final and definitive.

Despite these drawbacks, we feel that certain advances have been made. These are:

1) The three major components EF, PA, and LF have been partially purified and freed of all extraneous antigens for which tests were made. The use of these characterized components for studies on the mechanism of action of the toxin will greatly further our knowledge in this area.

2) The molecular weights of PA and LF have been estimated to lie between 60,000 and 100,000.

3) The studies on chemical bonding of the individual components indicate that PA and LF depend upon hydrogen bonds for their molecular configuration and antigenic activity while EF is not dependent on hydrogen bonds.

4) The configuration necessary for biological activity of the components appears to be different from that needed for serological and most likely antigenic activity.

5) The fact that dissociation occurs with both in vivo and in vitro produced toxins under favorable storage conditions makes it imperative that the techniques for both field and laboratory work be standardized and closely followed.

REFERENCES


Pathophysiological and biochemical changes in anthrax

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AND FREDERICK KLEIN

Process Development Division, U.S. Army Biological Laboratories,
Fort Detrick, Frederick, Maryland

Anthrax is a disease in which, as repeatedly mentioned in the literature, the signs or symptoms are not consistent with the severity of the disease and nearness of death.

Lack of marked specific pathophysiological changes may well be the reason why no extensive physiological study was made during the long era of the histopathologist.

In 1954 Smith, Keppie, and Stanley (5) demonstrated that Bacillus anthracis produced toxin in vivo and later showed that toxin could be produced in vitro. As work in this area increased, better tests for toxin were developed and coincidentally equipment was transistorized and developed as a routine tool for physiological monitoring of host responses.

In 1960–1961, when our group started its research on anthrax pathogenesis, there were several hypotheses for the cause of death: 1) capillary blockage by bacilli (6), 2) kidney shutdown and progressive secondary shock (5), 3) attachment of toxin to WBC and destruction of RES cells (1), and 4) altered capillary permeability and hypotension which perhaps is the basis of the more recently proposed pulmonary edema (2). We have subsequently suggested central nervous system (CNS) depression and respiratory failure as the cause of death (4).

We will review the pathophysiological data from the standpoint of 1) changes in blood cellular elements, 2) changes in blood gas and chemical constituents, 3) other signs and symptoms, and 4) other physiological changes. We shall, when possible, compare the infectious disease with that caused by the sterile toxin of B. anthracis.

A review of the literature showed that a number of biochemical parameters had been investigated, yet it still was impossible to identify the system or systems affected which resulted in the death of the host. There was general agreement that white blood cell (WBC) count increased and that pO2 decreased markedly. There was little to indicate that the disease caused by the organism or toxin was different. We measured a number of physiological parameters in spore- and toxin-challenged rhesus monkeys, chimpanzees, and rats to demonstrate whether there was similarity of response among species and whether toxin alone would produce changes similar to those observed during infection, and hopefully, give us a clue to the system or systems primarily affected.

Five rhesus monkeys and four chimpanzees were challenged by either the aerosol or intradermal route with spores and the course of the disease characterized. Each animal served as its own control in that it was monitored several hours before challenge to establish base-line values. The blood cellular responses (Figs. 1 and 2) are shown as statistically fitted lines. Increases in WBC counts and hematocrit occurred very late in the course of disease. We observed (Fig. 3) that blood glucose and calcium were decreased, while total protein remained in normal limits. During septicemia some monkeys developed a precipitous fall in blood glucose and died with terminal blood glucose levels of approximately 30 mg/100 ml. The blood chemical parameters assayed in the chimpanzees were glucose and the electrolytes Na+, K+, and Cl− (Fig. 4). The precipitous fall in glucose level was again seen with some animals showing levels of only 10 mg/100 ml terminally. The Na+ also decreased while K+ and Cl− increased terminally. The increased K+ is partly attributable to lysis of erythrocytes and somatic cells. Blood pH and gas changes monitored on the same animals indicated two systems were involved (Table 1). A respiratory alkalosis occurred in early septicemia then terminally a metabolic acidosis was superimposed.

The responses of seven rhesus monkeys challenged intravenously with toxin were essentially identical with those observed for the spore-challenged animals; however, the changes occurred relatively earlier in the course of disease. Phosphorus showed a slight increase in concentration (Figs. 5 and 6).

The decreased calcium concentration probably is the result of increased pH because the ratio between protein-bound calcium and free calcium is pH dependent. The changes observed in calcium and potassium levels allow us to explain the hyperesthesia and muscle spasms seen in some humans and animals dying of anthrax as neuromuscular irritability, which is enhanced
observed by us in the primates and by others in rabbits, rats, and sheep led us to follow this in the Fischer 344 rat. When rats were challenged with whole toxin or its lethal factor (LF) and protective antigen (PA) components combined (Table 2), not only was serum glucose lowered by either a decrease in calcium or increase in potassium. In anthrax, both changes occur. It is probable that more humans and animals do not display this clinical syndrome because of the extreme hypoxia and hypoglycemia which occur. Whether or not a quiet death or a violent one occurs depends on which of the above systems is altered the most at the time of death. The blood cellular responses in the rabbit were identical to those of the monkey and chimpanzee for both toxin and spore challenge. The dramatic changes in blood glucose levels

**Fig. 1.** Individual and mean blood cellular values of five monkeys following aerosol or intradermal challenge with the VIB strain of Bacillus anthracis. Relative time is a mathematical expression of actual responses to allow presentation of the data. (From Klein et al. with permission of J. Infect. Diseases (3).)

**Fig. 2.** Individual and mean blood cellular values of four chimpanzees following aerosol challenge with the VIB strain of Bacillus anthracis. Relative time is a mathematical expression of actual responses to allow presentation of the data. (From Klein et al. with permission of J. Infect. Diseases (3).)

**Fig. 3.** Individual and mean blood chemical values of five monkeys following aerosol or intradermal challenge with the VIB strain of B. anthracis. Relative time is a mathematical expression of actual responses to allow presentation of the data. (From Klein et al. with permission of J. Infect. Diseases (3).)

**Fig. 4.** Individual and mean blood chemical values of four chimpanzees following aerosol challenge with the VIB strain of B. anthracis. Relative time is a mathematical expression of actual responses to allow presentation of the data. (From Klein et al. with permission of J. Infect. Diseases (3).)
TABLE 1. Blood pH and gases in uninfectcd and infected chimpanzees

<table>
<thead>
<tr>
<th></th>
<th>Prechallenge</th>
<th>Postchallenge</th>
<th>Septicemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>pO₂</td>
<td>47.5</td>
<td>47.7</td>
<td>27.5</td>
</tr>
<tr>
<td>pCO₂</td>
<td>47.2</td>
<td>43.2</td>
<td>39.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.39</td>
<td>7.49</td>
<td>7.45</td>
</tr>
</tbody>
</table>

FIG. 5. Individual and mean blood cellular values of seven monkeys following intravenous challenge with Bacillus anthracis toxins. Relative time is a mathematical expression of actual responses to allow presentation of the data. (From Klein et al. with permission of J. Infect. Diseases.)

MEAN RELATIVE TIME IN HOURS DURING THE COURSE OF THE DISEASE

to hypoglycemic levels, but a decrease in liver and muscle glycogen followed in that order. These extremely low glucose and glycogen levels observed in toxin-challenged rats also occurred in spore-challenged rats.

The monkey and chimpanzee were used for further physiological studies. Monkeys were challenged either by spores or toxin while the chimpanzees were challenged only with toxin. The parameters monitored were EEG, heart rate, EKG, respiration, phrenic nerve discharge, and blood pressure. Depression of the EEG and a decrease in the respiratory rate occurred within 5–8 min after injection of 10,000 units of toxin in the rhesus monkey and 100,000 units of toxin in the chimpanzee (Fig. 7). One-third of the animals became comatose from which state recovery was rapid. The EEG then remained essentially normal until a few hours before death although a cyclic type of depression was observed in some animals. The response of one animal which is typical of the group is shown in Fig. 7. At 26 hr post-challenge, respiration ceased. Death was preceded by 6 hr of grossly abnormal EEG, which developed into a progressive depression terminating in complete silence. Cessation of respiration was followed by anoxic myocardial failure. At death, stimulation of the peripheral end of the cut phrenic nerve elicited a hyperreactive response of the diaphragm indicating that with anthrax intoxication there is no block of the neuromuscular transmission such as has been observed with snake ven-

ome and botulinum toxin. It appeared rather, that the brain was depressed and no longer capable of initiating an electrical discharge. Spore-infected monkeys displayed a similar EEG pattern (Fig. 8). In both toxin- and spore-challenged monkeys the EKG, blood pressure, and respiratory rate did not deviate from the normal range until shortly before death. In all cases the heart con-

FIG. 6. Individual and mean blood chemical values of seven monkeys following intravenous challenge with B. anthracis toxins. Relative time is a mathematical expression of actual responses to allow presentation of the data. (From Klein et al. with permission of J. Infect. Diseases.)

MEAN RELATIVE TIME IN HOURS DURING THE COURSE OF THE DISEASE

TABLE 2. Changes in glucose and glycogen levels following challenge with anthrax spores or toxin

<table>
<thead>
<tr>
<th>Time</th>
<th>Serum Glucose, mg/100 ml</th>
<th>Tissue Glycogen, mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Leg</td>
</tr>
<tr>
<td>Lethal preparations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spores</td>
<td>0 hr</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>16 hr</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>94 hr</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>0 min</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>80 min</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>140 min</td>
<td>64</td>
</tr>
<tr>
<td>Whole toxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>77</td>
<td>131</td>
</tr>
<tr>
<td>80 min</td>
<td>66</td>
<td>125</td>
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<tr>
<td>140 min</td>
<td>98</td>
<td>17</td>
</tr>
<tr>
<td>Protective antigen + lethal antigen</td>
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<td></td>
</tr>
<tr>
<td>0 min</td>
<td>73</td>
<td>131</td>
</tr>
<tr>
<td>80 min</td>
<td>50</td>
<td>71</td>
</tr>
<tr>
<td>140 min</td>
<td>83</td>
<td>41</td>
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<tr>
<td>Nonlethal preparations</td>
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</tr>
<tr>
<td>Protective antigen</td>
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<tr>
<td>0 min</td>
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<td>131</td>
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<td>80 min</td>
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<td>91</td>
<td>62</td>
</tr>
<tr>
<td>140 min</td>
<td>73</td>
<td>38</td>
</tr>
</tbody>
</table>

* These rats had not been prestarved for 24 hr as the others had. These data were not included in the statistical analysis.
Fig. 7. Effect of anthrax toxin on EEG, heart rate, EKG, respiration, and phrenic nerve discharges in the anesthetized monkey. (Unpublished data.)
changes and all animals survived. However, if administered after 8 hr, or at one-third of the time required for death of the untreated controls, the reversal was not observed and the animal died with an extended time to death.

Of the three components of toxin it was determined that protective antigen caused the initial EEG changes. When purified PA is injected intravenously, immediate changes in EEG and other parameters were observed similar to those following administration of whole toxin; however, all animals rapidly recovered. Purified lethal factor (LF) alone did not cause changes in the parameters we measured, and all animals survived. When PA was injected, followed in 30 min with LF or vice versa, the same physiological changes were observed as were seen with whole toxin. Specific control for this system was shown when one lot of purified LF and PA which had lost its activity upon lyophilization produced no demonstrable changes, and therefore ruled out the possibility that protein, salts, and the like, contained in the preparations were causing the observed changes. Toxin inactivated with antisera produced no changes in physiological parameters being monitored.

When as little as 700 units of toxin were injected into the cerebral spinal fluid via the cisterna magna, monkeys died in 6–10 min. In all deaths from toxin the heart continues to beat after respiration ceases. Flow rates and blood pressure from the left side of the heart fall sharply and remain low following toxin administration (Fig. 9). Necropsy substantiates that the right side of the heart, pulmonary artery, and vena cava are engorged and distended, while the left side is virtually empty.

The model depicting the disease syndrome published by us in 1964 thus has withstood the test of experimentation. This model was constructed from the reports in the

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**FIG. 8.** EEG tracings of the monkey prechallenge and during lethal anthrax infection. (Unpublished data.)

**FIG. 9.** Aortic flow rate of the rhesus monkey after challenge with 1,000 units of *Bacillus anthracis* toxins (subdural injection into cerebrospinal fluid). (Unpublished data.)
literature on sudden death in all species, including man. Disregarding cutaneous lesions, the individual exhibits few symptoms or signs until just before death. Also, our observations including hyperactivity and spastic paralysis in numerous species of animals dying of anthrax tended to support this observation. Most changes reported in the literature and our own findings can be explained as primarily a CNS involvement. The cellular, biochemical, and physiological alterations appear to be secondary or nonspecific and contribute to an unknown degree in the death of the host.

In conclusion we can make several points:

1) There is no doubt that the disease syndrome is caused by the toxin and not the organism per se. This knowledge follows the classical work of the Porton group in identifying the toxin, the results of which make anthrax what one might reasonably consider an etiologically new disease, with many similarities to diphtheria and tetanus.

2) From our observations on several species of animals we conclude that death is caused by toxin acting on the CNS with depression and paralysis of the respiratory center. Several other parameters also are affected and at death an extreme anoxia, hypoglycemia, and alkalosis develop. A decreased Ca++ and an increased K⁺ and Cl⁻ were observed. White blood cell counts and hematocrit increased with cardiac changes occurring just before respiration ceases.

3) The pathophysiological changes occurring during the infectious disease or during the toxemic disease are remarkably similar, although an occasional difference, as for example, pulmonary edema, is noted in the rat.

The application of these findings in the treatment of anthrax will be considered in later discussions.

REFERENCES


Epinephrinelike activity of culture filtrate from *Bacillus anthracis*

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The events leading to death of animals infected with anthrax are not clear (1, 19, 20, 23, 27). Severe alterations in circulatory dynamics (1, 23, 27) and in blood chemistry occur (4, 12, 19, 26, 27). Nordberg et al. (23) found in rabbits, during the initial phase of the septicemia, normal or elevated blood pressure that persisted until just prior to death. The animals then went into profound, irreversible shock. Stein and Logan (26), Eckert and Bonventre (12), and Klein et al. (19) reported an initial hyperglycemia following intravenous administration of filtrates obtained from cultures of *Bacillus anthracis*. Stein and Logan attributed this hyperglycemia to release of endogenous epinephrine by anthrax toxin since pretreatment with ergotamine blocked the rise in blood glucose. In these three studies, all of the filtrates contained anthrax toxin. The possibility that the filtrates themselves might possess an epinephrinelike activity was not suggested.

We wish to present evidence that crude filtrates from cultures of *B. anthracis* contain a substance that mimics the vascular effects of epinephrine when injected intravenously into pithed rats; that the response is mediated through an effect on the alpha- and beta-receptors of the autonomic nervous system in a manner similar to that of epinephrine; and that this effect is produced by an inherent property of the filtrate itself rather than by an ability of the filtrate to release endogenous catecholamines.

Materials and Methods

A rough variant of the Vollum strain of *B. anthracis* (25) was grown in the chemically defined, liquid medium of Thorne et al. (30). Before autoclaving, the medium was adjusted to pH 7.5 with 10% NaOH. The bicarbonate, sugars, and thiamine were dissolved in the same solution and sterilized by filtration. The FeSO₄ was also sterilized separately by filtration. These solutions were added aseptically to previously autoclaved medium.

The inoculum was prepared by suspending in 3–5 ml of 0.9% saline the growth on a slant of tryptic soy agar (Difco) incubated overnight at 37 C. About 1 ml of the saline suspension of organisms was inoculated into 250 ml of medium contained in a 2-liter flask. The flask was closed with a rubber stopper, and the cultures were incubated with shaking for 24 hr at 37 C. The cells were then removed by filtration through an asbestos filter (Sefit). The filtrates were stored overnight at 4 C to precipitate salts, lyophilized, and refrigerated. The yield from 1 liter of culture filtrate was approximately 20 g of solid material. The white powder was weighed and dissolved in isotonic saline for intravenous injection. This solution was used to make further dilutions for biological assay.

Male albino rats weighing 200–300 g were anesthetized with ether, pithed and placed immediately on artificial respiration (32). A carotid artery was cannulated and the blood pressure was recorded. Test substances were administered by tail vein in a volume of 0.2 ml of isotonic saline.

Results

Injection of epinephrine (Fig. 1a) produced a marked elevation in both systolic and diastolic pressure following which the diastolic pressure dropped, leaving a widened pulse pressure. The effect of intravenous injection of the crude anthrax filtrate (Fig. 1b) is similar, except for an initial transient drop in blood pressure. Injection of autoclaved medium alone (Fig. 1c) produced no effect other than that attributable to the volume of the injection. Injection of a similar quantity of filtrate obtained from cultures of *Escherichia coli* (Fig. 1d) grown in the same medium and prepared by the same method as used for the anthrax filtrate did not evoke a response like that obtained with material from *B. anthracis* (Fig. 1b).

These preliminary experiments suggested that the material mimicked the vascular effects of epinephrine when injected into pithed rats and prompted an investi-
FIG. 1. Effect on carotid blood pressure of intravenous injection into pithed rats of: a) epinephrine (50 μg); b) filtrate (15 mg) from B. anthracis; c) autoclaved medium alone (15 mg); and d) filtrate (15 mg) from E. coli grown in same medium as b. Arrows indicate time of injection, figures on ordinate, blood pressure in millimeters of mercury and distances between pips on abscissa, time in minutes.

Figures a-d illustrate the response to agents such as tyramine that release endogenous catecholamines but potentiate the response to exogenously administered epinephrine and norepinephrine (6, 13, 28, 34). In a pithed rat given intravenous cocaine 20 min prior to assay, tyramine produced no response (Fig. 3a and b). The response to epinephrine and to the anthrax filtrate (Fig. 3c and d) was markedly potentiated following treatment with cocaine (Fig. 3e and f).

Prior treatment of an animal with reserpine effectively depletes the animal of peripheral stores of catecholamines (5, 34). Adrenalectomy removes the sources of epinephrine and norepinephrine present in the adrenal medulla. An adrenalectomized rat was treated with intraperitoneal reserpine 24 hr prior to assay. The animal was

Phentolamine, a specific alpha-receptor blocking agent, prevents the vasoconstriction caused by epinephrine (33). Treatment of a pithed rat with intravenous phentolamine 2 min prior to intravenous administration of epinephrine (Fig. 2a) and of anthrax filtrate (Fig. 2b) completely blocked the expected rise in systolic pressure. The fall seen in both systolic and diastolic blood pressure is due to unopposed beta-receptor stimulation.

Pronethalol, a new adrenergic blocking agent, effectively blocks beta-receptor stimulation (3). Prior treatment of a pithed rat with pronethalol prevents the biphasic response normally produced by epinephrine (32). Treatment of a pithed rat with intravenous pronethalol 20 min prior to intravenous administration of epinephrine and of anthrax filtrate completely blocked the expected fall in diastolic pressure (Fig. 2c and d). Only a pressor response is demonstrated that is the result of unopposed stimulation of the alpha-receptors.

Simultaneous use of both phentolamine and pronethalol almost completely abolished the effect of epinephrine (Fig. 2e) and of the anthrax filtrate (Fig. 2f) upon the blood pressure.

To determine whether the filtrate acted by releasing endogenous epinephrine or if it possessed intrinsic epinephrinelike activity, cocaine was used. Cocaine abol-

FIG. 2. Effect of phentolamine and pronethalol upon carotid blood pressure of pithed rats injected intravenously with epinephrine (50 μg) or anthrax filtrate (15 mg). Phentolamine (1 mg/kg) was given intravenously 2 min prior to challenge with a) epinephrine or b) anthrax filtrate. Pronethalol (1 mg/kg) was given intravenously 20 min prior to challenge with c) epinephrine or d) anthrax filtrate. Phentolamine (1 mg/kg) and pronethalol (1 mg/kg) were given simultaneously prior to challenge with e) epinephrine or f) anthrax filtrate. Other designations the same as in Fig. 1.
fully depleted of endogenous catecholamines since tyramine (Fig. 4a) produced no response. The filtrate (Fig. 4b) still caused a rise in both systolic and diastolic pressure, thus indicating that the activity resided in the filtrate itself.

**DISCUSSION**

The effects of bacterial products upon the physiology of the host have not been clearly defined and the possibility that such products might possess specific endocrinological activity has been little explored. Nelson et al. (22) and Chedid and Boyer (7-9) reported production by bacteria of substances with adrenocorticotropic hormonal activity. Among other criteria used in these studies, both groups used depletion of adrenal ascorbic acid in hypophysectomized rats as a test of adrenocorticotropic activity. The nonspecificity of this indicator has been emphasized (21). Danowski et al. (11) in reporting a case of Cushing's syndrome associated with nocardiosis suggested that the organism might produce ACTH or corticosteroids. Crawford et al. (10) found that the rise in steroids produced by injection of a lysate of *Nocardia asteroides* into intact animals was abolished by hypophysectomy. They concluded that the lysate acted as a "nonspecific stressor" that caused release of endogenous ACTH. Diphtherial toxin was also found to be such a nonspecific stressor by Hilgers et al. (18).

The epinephrinelike activity reported here is an intrinsic property of the filtrate. The activity is not directly associated with the "toxin" of *B. anthracis* since conditions necessary for toxin production were omitted (1, 2, 16, 17, 31), and massive doses of our filtrate (0.2 g) did not cause death in the assay animals as has been reported with toxic filtrates (1, 2, 13, 16).

Evaluation of the role this substance might play in the pathogenesis of anthrax must be determined. However, certain features of the experimental disease (12, 19, 20, 23, 26) are compatible with that produced by abnormally elevated levels of circulating catecholamines. Slein and Logan (26) attributed the hyperglycemia to release of endogenous epinephrine by the toxin. Our findings suggest that the filtrate itself possesses epinephrinelike activity. Indeed, the blood pressure changes seen in anthrax septicemia are very similar to those produced by prolonged intravenous infusion of epinephrine or noradrenaline (14, 15, 35), with an initial elevation of blood pressure followed by hyporeactivity and profound terminal hypotension.

The importance of the catecholamines in the pathogenesis of bacteremic shock is recognized (24, 29, 36). Zweifach et al. (36) demonstrated that rats given endotoxin from gram-negative bacteria were hypersensitive to subsequent intravenous doses of epinephrine. A vasosconstriction of the terminal arterioles and venules persisted until the vessels gradually became hyporeactive and dilated, resulting in terminal shock. We would emphasize that anthrax filtrate did not potentiate the effect of exogenously administered epinephrine but
rather possessed an epinephrinelike activity of its own. We believe that the discovery of a bacterial product with intrinsic epinephrinelike activity may be of importance in explaining some of the pathophysiological events leading to death in anthrax and possibly other bacterial septicemias.

REFERENCES

Attempts to implicate the central nervous system as a primary site of action for *Bacillus anthracis* lethal toxin

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The basic mechanisms by which the toxin (lethal factor) produced by *Bacillus anthracis* contributes to the pathogenesis of anthrax are not known. The extracellular toxin which the organism synthesizes both in vitro (17) and in vivo (18) has been shown to be a complex molecular entity made up of three components, all of which seem to be necessary for complete biological activity to be expressed. This situation is not unique since multi-component toxins are produced by other bacterial species. For example, the staphylococcal leukocidin is made up of two protein components, neither of which is biologically active by itself (19). The components of the anthrax toxin have been designated as Factor I (edema factor), Factor II (protective antigen) and Factor III (lethal factor). In spite of the fact that some progress has been made in the purification of the components (7), knowledge concerning the chemical nature of the toxin is incomplete at this time. The two considerations which have impeded biochemical characterization are the extreme lability of the toxin and the lack of a simple, reliable assay system for its detection.

During the past several years, much experimental evidence has been accumulated which suggests that the anthrax toxin contributes significantly to the pathophysiology of the infection. The anthrax toxin is lethal for several animal species when administered parenterally although the order of magnitude required to cause death is much greater than for the classical exotoxins. The Fischer 344 albino rat has been found to be extremely sensitive (2). Since this strain of rat succumbs within several hours or less, it has been adopted for assay of the toxin (10). Other strains of rat, the guinea pig, and the rhesus monkey have also been used in studies of the lethal properties of the anthrax toxin (11). Studies employing the primates and the Fischer rat have been the most definitive in establishing the importance of the toxin as a cause of death during anthrax infection. These studies have also shown that the toxemia mimics anthrax infection in many respects and suggest that the central nervous system may be a primary site at which the toxin acts (12). The most significant observations implicating the central nervous system are the following: 1) the Fischer rat dies with severe pulmonary edema following administration of the toxin (2, 3) and the identical pathological change has been observed in a large percentage of rats following the induction of lesions in the hypothalamus with electrical current (14); 2) when a lethal dose of the toxin is injected intravenously, both rhesus monkeys and rabbits develop a spastic paralysis of the forelimbs several hours before death; 3) all animals tested develop a hyperreactivity to sound and touch in the terminal stages of the infection or toxemia; 4) the electrolyte imbalance, hypoglycemia, and hypoxic blood which are characteristically observed during the terminal stages of anthrax infection or toxemia are consistent with a disruption of the integrated activities of the hypothalamus and adrenal cortex (9); and 5) electroencephalographic patterns in monkeys become abnormal during the anthrax toxemia. The evidence suggests that death is due to respiratory failure of central nervous system origin (Lincoln, Vick and Klein, unpublished results).

As far as we were able to ascertain, there are only two reports in the literature concerning central nervous system histopathology due to anthrax infection and the reports are contradictory. De Moulin (5) in an extensive study with several species of domestic animals concluded that severe pathological changes in the central nervous system occurred as a result of anthrax infection. He demonstrated widespread neuronal degeneration in several species of domestic animals which had died of anthrax septicemia. Smith and Keppie (15), however, reported that Ross in a histological survey of tissues obtained from guinea pigs dead of untreated anthrax infection had not observed any of the central nervous system changes described by de Moulin. Our report
describes attempts to resolve these conflicting observations and to expand the experimental procedures so that conclusions would not be dependent exclusively on histological evidence. This paper describes several types of experiments designed to establish whether or not the CNS may be directly involved as a primary site of the anthrax toxin’s biological activity. The first series of experiments represents a comprehensive histological examination of central nervous system tissue obtained from rhesus monkeys and Fischer rats that had expired either as a result of anthrax infection or intravenous infusion of in vitro produced anthrax toxin. The prior studies cited (5, 15) dealt only with anthrax infection. The second series of experiments was carried out to determine if inoculation of the toxin directly into the central nervous system circulation via the carotid artery increased its lethal effect; or if the toxin had any observable effect on the “blood-brain barrier” as previously described for endotoxin (6), snake venoms (16), and various other drugs (4).

MATERIALS AND METHODS

Animals. Rhesus macaca monkeys were conditioned at the Fort Detrick animal farm for 3 months prior to use and were found to be free of tuberculosis and other infectious diseases. The animals were estimated to be 4 years of age and approximately 12 lb. each.

Fischer 344 rats were obtained from the Charles River Farms, Massachusetts, or Microbiological Associates, Bethesda, Maryland. Animals of either sex weighing between 200–400 g were employed.

Anthrax toxin. The preparation employed throughout the investigation was a lyophilized crude toxin which consists essentially of a sterile culture filtrate of the avirulent Sterne strain of B. anthracis. The toxin was prepared by the method of Haines, Klein and Lincoln (10) and stored at −70 C until used. It was reconstituted in sterile distilled water to the desired concentration immediately before injection.

Preparation of central nervous system tissue for histological examination. A total of five monkeys was used in the experiment. Three were challenged with lethal quantities of the anthrax toxin intravenously, one was given a lethal dose of virulent B. anthracis spores intramuscularly, and the other served as a culture medium control, i.e., the same culture medium used to prepare the toxin was injected intravenously to insure that the un inoculated medium per se did not produce any pathological changes. Table 1 describes the details of the experimental procedure up to the time of death, or sacrifice of the monkeys. Cardiac perfusion with buffered formalin was carried out immediately upon death to insure adequate and rapid fixation of the central nervous system tissues.

Rats were injected with 1.0 ml of single strength toxin via a tail vein. Death occurred within several hours and pulmonary edema was used as an index of anthrax toxinemia. Central nervous system tissues were fixed immediately after death by cardiac perfusion with buffered formalin.

<table>
<thead>
<tr>
<th>Material Injected</th>
<th>Dose and Route of Injection</th>
<th>Volume Injected, ml</th>
<th>Time to Death, hr</th>
<th>Time to Sacrifice, hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthrax toxin</td>
<td>8,000 units (iv)†</td>
<td>25</td>
<td>70</td>
<td>89.5</td>
</tr>
<tr>
<td>Anthrax toxin</td>
<td>7,000 units (iv)</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthrax toxin</td>
<td>10,000 units (iv)</td>
<td>25</td>
<td>73.5</td>
<td></td>
</tr>
<tr>
<td>B. anthracis spores</td>
<td>1.2 X 10^6 (intr)</td>
<td>1.0</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Sterile culture medium</td>
<td>(control)</td>
<td>45</td>
<td></td>
<td>77</td>
</tr>
</tbody>
</table>

* Central nervous system tissues fixed by cardiac perfusion with buffered formalin at time of death or sacrifice of the animal. † Units of anthrax toxin are calculated from a regression line correlating the reciprocal of the response time of Fischer rats and the potency of the toxin preparation (10).

Sectioning and staining of central nervous system tissues. All brains and spinal cords of the monkeys and brains of rats on external examination and on sectioning at 1.0 cm levels showed no noteworthy abnormalities. Blocks of tissues were taken from right and left cerebral cortex (frontal, temporal, parietal, occipital, and hippocampal), right and left basal ganglia, right and left thalamus, right and left hypothalamus, midbrain, midpons, right and left cerebral hemispheres, medulla, and spinal cord (cervical, thoracic, lumbar, and sacral levels). These blocks were passed through 70, 80, 95, and 100% alcohol and a chloroform-parafin mixture over a period of 3 days. Final embedding was in paraffin. The tissues were sectioned at 5–6 μ m on a rotary microtome (American Optical Co., New York). The following staining methods were used: hematoxylin and eosin, Kluever-Barrera (luxol fast blue and cresyl violet) for myelin, the Glee’s silver stain for axon cylinders, and phosphotungstic acid hematoxylin.

Surgical procedures for intracarotid injection of toxin. Rats were anesthetized by the intraperitoneal injection of 6 % chloral hydrate (0.5 ml/100 g body wt). An incision was made on the right side of the neck and the carotid arteries exposed. The external carotid artery was ligated and the toxin was injected into the common carotid artery. Ligation of the artery proximal and distal to the injection site was necessary to prevent leakage of blood through the puncture. This procedure allows the direct passage of the toxin to the central nervous system without shunting to other areas of the head and neck via the external carotid. Injections were made with a 27-gauge needle and in no case was a volume greater than 0.25 ml infused. In all cases, the material was injected slowly over a period of 30–60 sec to prevent damage to the blood vessels due to excessive pressures.

This surgical procedure was employed for two types of experiments.

1) Time-to-death comparison of carotid artery and tail vein injections of anthrax toxin. In these experiments the rats were injected with 0.25 ml of 4 X concentrated toxin in a carotid artery or 1.0 ml of single strength toxin via a tail vein.

2) Effect of anthrax toxin on the blood-brain barrier. Approximately 2 hr after injection of the toxin, when the
rats demonstrated dyspnea due to the development of pulmonary edema. 1.0 ml of trypan blue (2.6%) was injected into the tail vein. After 3–5 min the animals were sacrificed by cardiac perfusion with buffered formalin and the brains examined grossly and microscopically for evidence of staining by the dye. Positive staining was interpreted as evidence of disruption of the blood-brain barrier (6). Saline and endotoxin (500 μg) (Difco) were injected in lieu of the anthrax toxin as negative and positive experimental controls.

**RESULTS**

*Histological evaluation of monkey and rat central nervous system tissues.* The initial experiments were done to resolve the contradictions in the reports of de Moulin (5) and Smith and Keppie (15) regarding the presence or absence of central nervous system lesions as a result of anthrax infection, and in addition to evaluate the central nervous system effects of anthrax toxin. Of the three monkeys given anthrax toxin intravenously, one died 70 hr after and the other 73.5 hr after injection; the third was sacrificed at 89 hr after the administration of the toxin. In one animal infected by the administration of *B. anthracis* spores, the time to death was 55 hr. The control animal given sterile medium intravenously was sacrificed 77 hr later.

The brains and spinal cords of the monkeys and rats were processed as described in MATERIALS AND METHODS. Microscopically (in the monkey) there was no evidence in any of the areas of the central nervous system examined of acute neuronal death, acute swelling of oligodendroglia, or pathological changes in the astrocytes. The external and internal glial membranes, ependyma, and choroid plexuses were normal. Rarely, isolated instances of satellitosis and shrinkage of neurons were encountered in all five monkey brains but this was not in excess of that seen in normal rhesus monkey brains. There was no evidence of infection, and indeed, the only change was one of moderate congestion of the capillary network. The right and left hypothalami and the ninth and tenth cranial nerve nuclei were also examined. Again the results were essentially negative.

Examination of the central nervous system tissues of three Fischer 344 rats which had succumbed to a lethal dose of anthrax toxin also showed that no pathological changes occurred as a result of the fatal toxemia. In all three rats it was apparent that pulmonary edema had developed, as is the case in all Fischer rats injected intravenously with the anthrax toxin.

It would appear, therefore, that the absence of central nervous system lesions in both monkeys and rats is in agreement with the observations of Ross (15) who examined the central nervous system tissues of guinea pigs dead of anthrax infection and could find no significant pathological changes.

**Time to death of rats injected with anthrax toxin via carotid and intravenous routes.** The absence of demonstrable morphologic changes does not exclude the central nervous system as the primary site of anthrax toxin activity since biochemical lesions may not express themselves morphologically. A different approach focusing on the same question was adapted from the investigation of Porter and Kass (15). These investigators found that the lethal dose50 (LD50) of endotoxin administered via the carotid artery of rats was much less than the LD50 of the endotoxin injected intravenously. They concluded from these observations that endotoxin probably acts in a region of the posterior hypothalamus. Using the same rationale and experimental model, anthrax toxin was tested in rats. In our experiments time to death rather than LD50 was used as the assay. Table 2 shows that the animals injected via the carotid artery or via the intravenous route (tail or femoral vein) succumbed to the anthrax toxin within the same length of time. If the primary locus of action of anthrax toxin is in the central nervous system, then we would expect that the animals receiving the intracarotid injections would expire sooner than those injected intravenously. Eleven rats received anthrax toxin intravenously and the time to death averaged 146 min. The average time to death of the 15 that received anthrax toxin via the carotid artery was 162 min. In
TABLE 3. Penetration of trypan blue into the central nervous system of Fischer rats injected with anthrax toxin via a carotid artery

<table>
<thead>
<tr>
<th>Time to Death, min</th>
<th>Pulmonary Edema</th>
<th>Trypan Blue Staining of Brain Tissues*</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>65</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>110</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>127</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>120</td>
<td>+/-</td>
<td>–</td>
</tr>
<tr>
<td>60</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>70</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>102</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

* Each animal was injected with 0.25 ml of 4 × concentrated anthrax toxin into a carotid artery. After development of overt dyspnea 1.0 ml of trypan blue (9.0%) was injected intravenously. † Staining of the meninges was considered negative. A positive reaction was recorded only if the brain tissues per se were stained.

both of these groups, the action of anthrax toxin was confirmed by the presence of grossly visible pulmonary edema. The lungs of the animals dying of pulmonary edema weighed between 1.5 and 3.0 g, and red frothy fluid could be expressed from the cut surfaces in each lung.

The brain of each animal in both groups was examined and no gross morphologic lesions could be observed either externally or on multiple coronal sectioning.

Effect of anthrax toxin on blood-brain barrier of rats. Another means to relate the action of anthrax toxin to the central nervous system consisted of an attempt to demonstrate an effect on the blood-brain barrier. The model used here was adapted from the work of Eckman, King and Brunson (6), which is essentially a modification of the experimental procedure used in the previous experiments. Of the nine animals in which trypan blue was injected at varying intervals after anthrax toxin, the brains of two animals exhibited generalized bluish staining of the superficial cortex. The other seven animals exhibited no staining of the brains (Table 3). In each instance the successful introduction of dye was confirmed by the staining of the meninges by the dye. The successful introduction and sufficient time for action of anthrax toxin was confirmed by the presence of pulmonary edema. In almost every instance, the animals died within 3 min after the injection of the dye. The length of survival was dictated by the time we chose to inject the dye and not necessarily by the action of the toxin. The controls for this group of rats consisted of four rats receiving 0.25 ml of saline via a carotid artery followed by the injection of trypan blue intravenously (tail vein or femoral vein) at an interval of time approximating the interval between the toxin and dye injections. Although the visceral organs were stained, indicating passage of the dye into the systemic circulation, the brains remained unstained, thus ruling out significant demonstrable damage to the brain by introduction of fluid directly into the cerebral circulation. Since only two of nine anthrax toxin-treated animals gave evidence of damage to the blood-brain barrier, the results are equivocal and no definitive conclusion can be made.

DISCUSSION

A detailed histological examination of brain tissue obtained from rhesus monkeys and Fischer rats provided no evidence that the anthrax toxin produced visible pathological changes in the central nervous system of these two species. While the results are unequivocal they do not necessarily negate the possibility that the primary effect of the anthrax toxin is in the central nervous system. The absence of pathological changes in the brain tissues of the rat can be accounted for by the fact that the toxin acts very rapidly in this species. Invariably death occurs within several hours after injection of the toxin and it is not surprising that visible morphological changes were absent. In the case of the monkeys that survived toxin infusion for as long as 90 hr, however, this explanation would probably not suffice. It is clear that many central nervous system dysfunctions including those as serious as coma which often lead to death do not necessitate changes in the central nervous system tissues which can be visualized by light microscopy. An electron-microscopic evaluation might reveal pathological changes not made apparent by routine histological methods, but such a study was not attempted. We consider that the histological study was important in spite of the negative results obtained. It tends to resolve the conflicting reports of de Moulin (5) and Smith and Keppie (15), and shows rather conclusively that fatal anthrax infection or B. anthracis toxemia does not induce significant pathological changes in the central nervous system of two animal species. How far these results can be extrapolated to man or domestic animals remains conjectural.

Subsequent experiments with the Fischer rat not dependent upon histological evidence also failed to implicate the central nervous system as a primary site for the biological effects of anthrax toxin. Two types of experiments failed to enlarge upon the hypothetical central nervous system-toxin relationship. Porter and Kass (13) found that bacterial endotoxin probably acts on the central nervous system by establishing that the 1LD₉₀ in rats was reduced considerably when the endotoxin was administered via the carotid artery rather than intravenously. In similar experiments with anthrax toxin, such a relationship could not be established. The average time to death of rats injected intravenously and via the carotid artery was found to be essentially the same. Since time to death is a valid measure of anthrax toxin potency (10) the results suggest that inoculation directly into the central nervous system circulation does not enhance the biological potency of anthrax toxin.

Another possibility explored was that one or more components of the toxin altered the permeability of capillaries so that the effectiveness of the blood-brain barrier was impaired. This has been shown to be the case for endotoxin (6), snake venoms (16), and other bacterial toxins (4). Our results, however, were equivocal. Of a total of nine rats injected in the carotid artery with
toxin, followed by a vital dye injected intravenously approximately 2 hr later, only two demonstrated visible staining of the brain tissues. No definitive conclusion can be drawn from these data but they suggest that anthrax toxin may increase capillary permeability of central nervous system vasculature. That the toxin exerts this effect on lung and skin capillaries has been established conclusively (1). It would appear that these experiments should be repeated using a tagged protein (fluorescein or ferritin) rather than the colloidal particles of a vital dye which may be too large to traverse the capillaries in spite of increased permeability. It may also be possible to trace the toxin itself by means of radioisotope labeling or fluorescent antibody methods. This is now feasible since the multicomponent toxin can be purified as described by Dr. Fish (8).

REFERENCES

Anthrax toxic complex

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Work on anthrax has reached a point where the complexities of the problem can be seen and it may be worthwhile pausing to consider the questions which should be studied in the next phase of the investigation of this disease. Since the pathological changes in anthrax are attributed to the action of the extracellular “toxin” produced by Bacillus anthracis, many of these questions concern the nature and actions of the anthrax toxic complex.

It is now clear that the toxin contains three separate components, two proteins and a chelating compound containing phosphorus and with both protein and carbohydrate moieties (26, 28, 29). These components are not toxic when injected alone, but only when mixed together. There is some confusion about the behavior of various mixtures of the factors in toxicity tests and in tests for immunogenicity. Most of this confusion is due to the complexity of the system, but part is due to the different pathways by which the Porton and Fort Detrick groups recognized the three components. This has led to the use of different biological tests and also to different nomenclatures confusing to the nonexpert. The British group have called the components simply Factors I, II, and III which correspond, respectively, to the American names edema factor (EF), protective antigen (PA), and lethal factor (LF).

The fact that different workers have reported differences in biological behavior is not surprising in view of the complexity of the system. First of all, for significant tests, each factor must be free of the others. Then, not only must all three factors be tested alone at different concentrations but also their various combinations. In addition, the fact that not one but several biological tests have been used in this work does not help the situation.

The British group relied mainly on two biological tests for toxicity 1) edema production in the skin of guinea pigs or rabbits, and 2) lethality for mice. In tests for immunogenicity, guinea pigs and a spore challenge were used. Their conclusions are summarized in Table 1.

The American workers have used the two toxicity tests of the British group but in recent years have supplemented them by tests in Fischer 344 rats (2, 11). This animal is extremely sensitive to some toxic mixtures of the components from B. anthracis, dying with severe pulmonary edema 1–3 hr after injection compared with mice and guinea pigs which take 1–3 days to die. In tests for immunogenicity both guinea pigs and rats have been used with a spore challenge.

From toxicity tests both the British and American workers are agreed that all the factors are inactive when injected alone, that a mixture of Factor I (EF) and Factor II (PA) produces gross local edema and that a mixture of Factor II (PA) and Factor III (LF) is lethal when injected intravenously (2). The main difference in conclusions between the two groups concerns the effect of Factor I (EF) in toxicity tests on mice. The British group (28) believed that Factor I (EF) contributed materially to lethality of the mixtures since it formed a lethal mixture with Factor II (PA) and increased the lethality of mixtures of Factors II (PA) and III (LF). The American workers (2) assumed that Factor I (EF) did not contribute materially to the lethal potency of mixtures. They do not appear to have investigated another conclusion of the British group, namely that addition of Factor III (LF) to a mixture of Factors I (EF) and II (PA) decreased the production of local edema.

In recent tests for immunogenicity in guinea pigs and rats, American workers (18) have considered that Factor III (LF) was immunogenic when injected alone, as well as Factor II (PA). As regards the immunogenicity of various combinations of the three factors their complex results were not contrary to the main British conclusion that Factor I enhanced the immunogenicity of Factor II (PA). It is generally agreed that the best vaccine against anthrax should contain an optimum mixture of all three components and that serological methods using gel diffusion do not give a reliable estimate of degree of immunity (14, 22).

There is obviously fair agreement about many aspects of the biological testing of this complex system. The points of disagreement are probably not very serious and in seeking to explain them the following possibilities should be investigated: 1) The purity of the fractions being tested, e.g., could the immunogenicity of Factor
### Table I. Effect of isolated factors from the anthrax toxic complex in causing local edema after intradermal injection in skin of guinea pigs or rabbits and in causing death of mice after intravenous injection. Immune response to the three factors in guinea pigs is also shown.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Toxicity</th>
<th>Immunogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Edema</td>
<td>Lethality</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guinea Pig</td>
</tr>
<tr>
<td>I (chelating agent)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II (protein)</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>III (protein)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I + II</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>I + III</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>II + III</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>I + II + III</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

III (LF) reported by the American workers become due to contamination with Factor II (PA), or with Factor I (EF) which appears to produce an immunogenic mixture with Factor III (29). 2) The possibility of some animal species or strains being particularly sensitive to certain mixtures of components, e.g., the Fischer 344 rat to the mixture of Factors II (PA) plus III (LF). 3) The difficulty of covering adequately all possible combinations and concentrations of the three factors. 4) The possibility that some preparations of “whole toxin” are deficient in one or more components.

The advantages of the British system of nomenclature should be apparent from these discussions and those to follow on the pharmacology of the toxic complex. The system allows greater specificity and does not blur the similarities and differences between the components. The American system is confusing: for example, a mixture of Factor II (PA) and Factor III (LF) produces edema, particularly pulmonary edema (1, 2), as well as a mixture of Factor II (PA) and Factor I (EF)—the so-called edema factor; furthermore, the latter factor contributes to the lethal action of mixtures in mice as does Factor III—the so-called lethal factor (28).

In the further consideration of the pharmacology of the anthrax toxic complex and its relation to the pathology of anthrax two facts must be recognized at once. First, only the simplest toxicological studies have been made and a more sophisticated approach to these problems is needed, and second, almost all the work has been done with the crude mixtures of these components. The pharmacological activities of the individual components or the various binary mixtures have not been studied in detail. However, it is apparent that, like the whole toxic complex, a mixture of Factors I and II evokes the slow formation of local edema (28) and that a mixture of Factors II and III rapidly kills Fischer 344 rats with the formation of gross pulmonary edema (2).

The main effect of the anthrax toxic complex is to increase vascular permeability and this can be seen after either intradermal or intravenous injection. The slowness with which the edema forms after intradermal injection is of considerable interest. The standard permeability factors such as histamine and the kinins act rapidly and their effects are very short lived. At the present time, workers on inflammation are particularly interested in the more slowly developing changes in vascular permeability. This means that much more precise information is needed on the time course of the permeability changes after the intradermal injection of anthrax toxin at different dose levels. This could be studied by the dye techniques of Miles and Wilhelm (20) which often show that there are two phases to the permeability changes. Examples of the diphasic response seen after clostridial toxins are shown by Elder and Miles (9) and Logan and Wilhelm (17). In addition, the actual skin vessels involved in the response must also be defined using the carbon-labeling technique of Majno, Palade and Schöpf (19), coupled with electron microscopy (7). We should also know the time course of the cellular response to anthrax toxin given in this way.

Changes in permeability with fluid loss have also been produced by the intravenous injection of the toxic complex. Increasing vascular permeability by the intravenous, as opposed to the topical, application of a toxin is of special interest being a much less usual experimental situation. A severe loss of circulating fluid, comparable to that occurring in anthrax infection, was seen when crude anthrax toxin was injected intravenously in guinea pigs (24). After the intravenous injection of the toxic complex in the rat fluid loss occurs mainly in the lungs as pulmonary edema (1, 2, 8). This is best seen in one strain of rat, the Fischer 344, and most work has been done on this strain. Here the pulmonary edema develops much more rapidly than in other rat strains and leads to severe hypoxemia (8). It would be interesting to know the mechanism of this increased sensitivity. Does it represent some genetic difference in the Fischer 344 rat which could be transferred to other rats by selective breeding? It would be interesting also to know more about the other responses of this rat. Is it particularly sensitive to other agents which cause pulmonary edema such as α-naphthyl thiourea? Are these rats sensitive to dextran? Edema following the injection of dextran in rats is also genetically determined (12). The production of pulmonary edema by anthrax toxin in the Fischer rat might be usefully investigated in a perfused lung preparation of the type used by Born (4) where it would be possible to continue the study biochemically. Knowledge of the hemodynamic disturbances occurring in this condition would also be useful. From the recent work of Beall and Daldorf (1) showing the very high protein concentration (4.9-5.5%) in the edema fluid, it would seem certain that the toxin alters the permeability of the lung vessels. This does not necessarily mean that the toxin has a direct effect on the permeability of these vessels. Cheng (quoted by Cameron (5)) found that the protein content of the lung fluid was very high when edema was produced by such disparate methods as the intravenous injection of massive doses of adrenaline, or the intracisternal production of fibrin. Beall and Daldorf (1) also showed that the
toxic complex could increase vascular permeability at other sites in the rat, such as the peritoneal cavity and skin, but the changes developed much more slowly than in the lungs. (While these findings favor a direct action of the toxin on the vessels, experiments on the perfused lung are clearly necessary.) The effect of various drugs on these responses was observed and the results indicated that histamine and serotonin were unlikely to be concerned in the changes. They attempted to investigate the matter further by the injection of a carbon preparation and by electron microscopy, but their experiments in this respect are unsatisfactory as they did not follow the techniques of Majno and his colleagues (7, 19).

Two types of pulmonary edema should perhaps be mentioned in a discussion of this problem in anthrax, namely that due to adrenaline and that associated with nervous disturbances. Although adrenaline is released after the injection of anthrax toxin (23) the amounts required to produce pulmonary edema are very large (5, 6), too large to be produced by the rat under these conditions (27). The pulmonary edema associated with nervous disturbances was thought by Cameron (5) to be due to increased intracranial pressure. Although edema was easily produced in rats by the intracisternal production of fibrin, rabbits were equally susceptible and this type of edema has been reported in a number of species including man. Significant pulmonary edema after the injection of anthrax toxin has only been reported in the rat, especially in the Fischer 344 strain.

Most of the changes in the blood constituents, either in anthrax infections or after administration of toxin, are secondary to the lesion produced and are similar to those which occur in response to other forms of injury (8, 23, 25). However, it is not further work on the alterations in carbohydrate metabolism and the like which is required, but better quantitative descriptions of the lesions which give rise to these changes. Such descriptions are available in the cases of guinea pigs either infected or given crude toxin and of rats given crude toxin. In these cases the primary lesion appears to be increased permeability of blood vessels leading to fluid loss.

The main question would seem to be: how far can the effects of anthrax be attributed to fluid loss? The effects of fluid loss could be aggravated in some species and under certain conditions by respiratory embarrassment due to fluid loss into the lungs and by circulatory embarrassment due to pressure from fluid loss into the mediastinum. The sudden death which sometimes seems to occur in anthrax should not be lightly used as evidence against a fluid loss theory. Rapid death after a period of apparent well-being can occur in oligemic states at high environmental temperatures (16).

Further examination of the effects of the toxic complex and mixtures of the various factors on permeability may begin to throw light on the various differences between species and sites in anthrax infection. Large species differences in the response to permeability factors such as serotonin are well known (21). Since the toxins have not, so far, been found to have any enzymic actions (29) and have not been associated with any of the established permeability factors, their mode of action and the nature of any mediators which may be involved in it remain unknown.

A direct effect of anthrax toxic complex on the central nervous system has been postulated by some workers (15, 16). There does not seem to be any real evidence for this and no definite lesions could be found in the central nervous system of infected animals by Ross (quoted by Keppie et al. (13)).

It seems to be generally accepted now that death in anthrax is due to toxin. However, it is always a good thing to question such ideas from time to time and this question is raised in a recent paper by Ward et al. (36), describing death from anthrax in immunized guinea pigs. The complex nature of the toxin and the occurrence of different strains of the organism make this a difficult matter to investigate, but a detailed comparison of animals dying with and without circulating free toxin should help to decide if any features of the illness are not due to toxin.

Finally, there is the question of whether anthrax toxin can be used as a research tool. The hope of using a natural or synthetic toxic substance as a tool for the dissection of body mechanisms is the inspiration of much work in toxicology. Good examples of this are fluoracetate and dinitrophenol. The idea is not new being clearly stated by Claude Bernard in 1878 (3). Before one can do this, certain requirements must be met. The action of the toxic substance must be known in some detail and be reasonably specific. Obviously, the more specific and limited its action, the better from this point of view. The nature of the toxin should also be known otherwise there is no possibility of carrying out structure-activity studies which often form an important part of this type of work. At present anthrax toxin does not seem to fulfill these requirements. However, with the current interest in substances causing slowly developing permeability changes in blood vessels, the further study of the effect of the anthrax toxic complex on vascular permeability should yield information which will be useful in a general context.

REFERENCES

Value of field data for extrapolation in anthrax

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FREDERICK KLEIN, ALBERT J. ROSENWALD
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One of the unfortunate generalizations that we may make of the anthrax literature is that it is largely descriptive and almost completely lacks quantitative information. Even in laboratory experiments, animals are reported as unobserved for long periods of time, which makes any statements regarding the time of death or specific response at death questionable.

We will discuss experimental data leading to the hypothesis or model that species naturally fall into two classes: (a) those resistant to establishment of anthrax, but once established are susceptible to the toxin, and the converse situation; (b) species which are susceptible to the establishment of the disease but resistant to the toxin. The minimum data required which will allow a species to be placed into the category of resistant or susceptible to the establishment of anthrax will be indicated. Information that may be obtained from a serological survey is also discussed.

INFORMATION OBTAINABLE FROM DIRECT
OBSERVATION OF THE BLOOD DURING THE DISEASE.

Since data on the blood levels of bacilli and toxin at death are available, the relationship between these two variables will be presented, then more extrapolative aspects of this information will be discussed. In Table 1 are data showing that (a) each species has a characteristic rate of septicemic development, (b) death occurs when the number of bacilli in the blood reaches a predetermined number, and (c) the units of toxin are directly related to number of organisms per milliliter of blood. The septicemic doubling rate does not change due to changes in resistance attributed to immunity (guinea pig and rat); however, the number of organisms and units of toxin per milliliter of blood at death increases. Further information showing that the terminal number of organisms in the blood of guinea pig and rhesus monkey is directly related to the toxin level is given in Fig. 1. This relationship can be influenced by time of death (Fig. 2) in that the shorter the time to death, the higher the number of organisms and units of toxin per milliliter of blood at death, and conversely, the longer the time to death, the lower the number of organisms and units of toxin per milliliter of blood. The dose-response relation-

ship of the rat to sterile toxin (Fig. 3) also supports this generalization.

Certain other generalizations may be made from these data. We know that once a septicemia is observed progression of the disease is rapid and predictable. Figure 4 is modified after Keppie, Smith and Harris-Smith (5), and we have published similar data on several species. With the guinea pig, once the septicemia is detectable by observation of organisms on a blood smear, there is on the average 12 hr until death and about 4 hr in which treatment with streptomycin can be initiated with any expectation of recovery. Whether the host recovers or not depends upon the amount of toxin fixed. Keppie et al. (5) showed that elimination of the organisms after a critical level of about 1–3 × 10⁶ organisms/ml was reached, treatment with streptomycin, which reduced the level of organisms in the blood to essentially the zero level, merely extended the time to death. In both rats (6) and monkeys (7) challenged with sterile toxin, if antiserum was administered before a time which was about one-third of the period between challenge and death of the untreated control animals, the animals survived. If antiserum was given after this period, death still occurred; however, the time to death was extended. It is appropriate to remark that at this time, late 1966, the only way of either detecting or quantitating anthrax in vivo still is by direct observation of organisms in blood smears or by dilution plating of blood onto agar medium. The difficulty of observing organisms early in anthrax infection may not be fully appreciated. In our work on treatment, to quantitate the septicemic level at the time of treatment we relied on microscopic observation of the blood smears and a day later read our dilution plates for a more exact estimate of septicemic level. With a film 1 cm² which contained 0.0345 ml of blood, we could expect to observe only about three organisms in 150 microscopic fields when the number of organisms in the blood was 5,000/ml³. To make the positive statement with a 95 % probability of correctness that a density of only one organism per milliliter of blood was correct, about 3,000 fields would need to be observed. Of course, an organism might be observed in fewer fields, but not until 3,000 fields are searched without an organism being found can it be asserted with 95 % probability of correctness that
are demonstrable (Table 2). The data show that those species resistant to the establishment of anthrax are susceptible to toxin, whereas those susceptible to establishment are resistant to anthrax toxin. The conclusions drawn from data presented in Table 1 that animals having a high bacteremic level at death are resistant to toxin thus are supported. Some inferences as to dose relationships are given below.

NUMBER OF ORGANISMS REQUIRED TO ESTABLISH INFECTION

If one accepts the above basis for generalization, then the dose to establish anthrax infection is inversely related to the number of organisms per milliliter of blood at death. These data (Table 3) show that those species which require a large dose to establish the disease have a low number of organisms in the blood at death, and vice versa. Although we believe that the units of toxin are of

<table>
<thead>
<tr>
<th>Species</th>
<th>Doubling Time</th>
<th>Terminal Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>45</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>53</td>
<td>$1 \times 10^8$</td>
</tr>
<tr>
<td>Guinea pig (immune PA$_A$)</td>
<td>53</td>
<td>$5 \times 10^7$</td>
</tr>
<tr>
<td>Guinea pig (immune PA$_A$ + LV)</td>
<td>53</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Rhesus</td>
<td>48</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>155</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Rat, NIH black</td>
<td>120</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Rat, Fischer 344</td>
<td>120</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Rat, F 344 (immune PA$_A$)</td>
<td>102-139</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Rat, F 344 (immune PA$_A$ + LV)</td>
<td>102-139</td>
<td>$1 \times 10^6$</td>
</tr>
</tbody>
</table>

The density is less than 1/ml. This kind of data is valuable in interpreting the qualitative information scattered through the anthrax literature that G+ cells were observed with perhaps some adjective as “few” or “many” indicating the number present. When an occasional or few organisms are reported, we believe the number is about $10^4$/ml, almost certainly less than $10^6$/ml. When a large number of organisms is reported, the number likely is about $10^6$/ml ± 1 log. Why medical or veterinary bacteriology is not more quantitative remains a mystery since quantitation of the blood smear can be accomplished easily and observation of this smear can readily be recorded quantitatively. Not only would potential research information be accumulated, but a better prognosis and evaluation of the case could be made, which would allow for selection of the treatment indicated by the stage of development of the disease.

Nothing quantitative is recorded on the terminal level of organisms per milliliter of blood in man or most domestic animals (except one report on sheep of $1 \times 10^4$/ml (8)). Since we know that the minimum doubling time in any animal surely is greater than 50 min (the approximate observed values in the mouse and guinea pig), knowledge of the terminal level would allow one to estimate the probability of being able to detect anthrax in time to treat a host effectively. Those species having septicemia levels about $1 \times 10^5$ should be amenable to treatment; those with fewer than $10^7$/ml likely would be more difficult. The higher the level of organisms per milliliter of blood and the greater the doubling time, the greater the probability of detection and treatment of generalized anthrax.

FIG. 1. Interaction among the terminal variables in the immunized guinea pig and rat. Each point represents one immunization protocol and is the mean of 9-10 animals distributed among two populations.
more significance than the number of bacilli, these data are still more difficult to collect. Nevertheless, we have shown a strong positive correlation between these variables. May we emphasize that by obtaining quantitative information on the number of bacilli per milliliter of blood at death of any species of interest—man, cow, or buffalo—a calculated prediction can be made as to the probable dose required to infect that host. Criticism could arise from the fact that we do not know the route of infection in field cases; however, it is our experience with numerous laboratory species that the route of infection does not in any way affect the terminal level of organisms. In any case, we suggest that a calculated defendable dose is better than continued ignorance; therefore, until data become available this relationship is a reasonable working model. It is, moreover, a working

![Graph](image)

**FIG. 2.** Relation of terminal concentration of organisms in blood with time to death and terminal concentration of toxin in blood of control and immunized guinea pig. (Lincoln et al. 1964.)

**TABLE 2.** Relationship between susceptibility to toxin challenge and resistance to establishment of anthrax

<table>
<thead>
<tr>
<th>Species</th>
<th>Units of Toxin/kg Causing Death</th>
<th>Time to Death, hr</th>
<th>Relative Resistance to Parenteral Challenge of Spores*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>1,000</td>
<td>24</td>
<td>Very susc</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1,125</td>
<td>24</td>
<td>Susc</td>
</tr>
<tr>
<td>Rabbit</td>
<td>2,500</td>
<td>72</td>
<td>Susc</td>
</tr>
<tr>
<td>Rhesus</td>
<td>2,500</td>
<td>28</td>
<td>Susc</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>4,000</td>
<td>60</td>
<td>Susc</td>
</tr>
<tr>
<td>Rat, NIH black</td>
<td>280</td>
<td>20</td>
<td>Resistant</td>
</tr>
<tr>
<td>Rat, Fischer 344</td>
<td>15</td>
<td>2</td>
<td>Resistant</td>
</tr>
<tr>
<td>Dog (beagle)</td>
<td>60</td>
<td>20</td>
<td>Very resistant</td>
</tr>
</tbody>
</table>

* Specific information given in Tables 3 and 6.

![Graph](image)

**FIG. 3.** Regression of reciprocal response time of Fischer rats on log dose of anthrax toxins expressed in potency units.

**TABLE 3.** Inverse relationship between dose to establish anthrax and number of organisms/ml of blood at death

<table>
<thead>
<tr>
<th>Relative Resistance</th>
<th>Dose to Establish Parenteral Anthrax Spores</th>
<th>Species</th>
<th>Quantitation of Blood at Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very susc</td>
<td>5</td>
<td>Mouse</td>
<td>10^{6.9}</td>
</tr>
<tr>
<td>Susceptible</td>
<td>50</td>
<td>Guinea pig</td>
<td>10^{6.8}</td>
</tr>
<tr>
<td>Susceptible</td>
<td>5,000</td>
<td>Rabbit</td>
<td>10^{6.8}</td>
</tr>
<tr>
<td>Susceptible</td>
<td>3,000</td>
<td>Rhesus</td>
<td>10^{6.8}</td>
</tr>
<tr>
<td>Susceptible</td>
<td>1 X 10^6</td>
<td>Chimpanzee</td>
<td>10^{6.3}</td>
</tr>
<tr>
<td>Resistant</td>
<td>50 X 10^6</td>
<td>Rat</td>
<td>10^{4.6}</td>
</tr>
<tr>
<td>Very resistant ca.</td>
<td></td>
<td>Dog</td>
<td>15</td>
</tr>
</tbody>
</table>

**TABLE 4.** Susceptibility of leukocytes of several species to anthrax

<table>
<thead>
<tr>
<th>Species</th>
<th>Maximum Final Dilution of Positive Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig, cow, man, rabbit, sheep, horse</td>
<td>1:32</td>
</tr>
<tr>
<td>Mouse</td>
<td>1:16</td>
</tr>
<tr>
<td>Rat</td>
<td>1:4</td>
</tr>
<tr>
<td>Dog, swine</td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

model readily susceptible to critical experimental examination.

**TRANSLATION OF DISEASE MODELS**

Certainly the proposal which has been presented above is not the only approach to this problem. The ideal model would not result in the death of the host and since blood can be readily obtained, it would be a very desirable system on which to construct an extrapolative
model. Regarding anthrax, we know of only one other attempt to assemble facts so that some extrapolative evaluation may be made. Preliminary work has been presented by A. J. Rosenwald, W. I. Jones, Jr. and R. E. Lincoln (Extrapolation of Relative Susceptibility of Hosts to Anthrax, ms in preparation) on changes in phagocytic and anchractid activity of blood cellular components as influenced by anthrax toxin concentration. They attempted to extend the observations of Kashiba et al. (4) on the highest dilution of terminal guinea pig serum which gave positive inhibition of phagocytes from different species (Table 4). Those species having leukocytes most sensitive to toxin are several known to be susceptible to establishment of anthrax: the guinea pig, rabbit, mouse, and sheep with the unknowns, man, cow, and horse, included in this group; leukocytes of the more resistant species are very resistant to inhibition by toxin. Since Rosenwald and co-workers could not repeat the Japanese observations, they went on to survey other interactions between the phagocyte and spore. In Table 5 two responses that were studied are given. The differences among species are interesting, but until combined with other data (Table 6) have little consistency. When combined with the other quantitative information available on anthrax there is definitely a difference between the species listed as susceptible or resistant. In the

![Graph showing relationship between level of anthrax septicemia and time to death (guinea pig). Change in death time by streptomycin treatment. (From Keppie et al. (5)).]

### Table 5: Spores germinating intracellularly, phagocytes containing ≥ 20 organisms and phagocytes destroyed

<table>
<thead>
<tr>
<th>Species</th>
<th>% Germination within Phagocyte</th>
<th>% Phagocytes with ≥ 20 Cells</th>
<th>% Phagocytes destroyed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig</td>
<td>35</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>Guinea pig (immune PA₃)</td>
<td>29</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Rhesus</td>
<td>25</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>12</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>6</td>
<td>64</td>
<td>65</td>
</tr>
<tr>
<td>Man (immune PA₃)</td>
<td>0</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Rat, NIH black</td>
<td>17</td>
<td>28</td>
<td>70</td>
</tr>
<tr>
<td>Rat, Fischer 344</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat</td>
<td>68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swine</td>
<td>33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 6: Summary of data relating susceptibility in establishment of anthrax to other critical observations

<table>
<thead>
<tr>
<th>Terminal Blood</th>
<th>Susc Toxin Inhibition, dilution</th>
<th>Susc to Toxin Challenge</th>
<th>Phagocytes</th>
<th>Approx LD₅₀ Dose&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Parenteral</td>
<td>Aerosol</td>
</tr>
<tr>
<td>Susceptible sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>10⁵.⁸</td>
<td>1:32</td>
<td>V, Res</td>
<td>25</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>10⁶.³</td>
<td>1:32</td>
<td>Res</td>
<td>35</td>
</tr>
<tr>
<td>Rabbit</td>
<td>10⁵.⁶</td>
<td>1:32</td>
<td>Res</td>
<td>35</td>
</tr>
<tr>
<td>Mouse</td>
<td>10⁶.⁸</td>
<td>1:32</td>
<td>Res</td>
<td>25</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>10⁶.⁸</td>
<td>1:32</td>
<td>Res</td>
<td>0</td>
</tr>
<tr>
<td>Goat</td>
<td>High&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1:32</td>
<td>Res</td>
<td>6</td>
</tr>
<tr>
<td>Sheep</td>
<td>High&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1:32</td>
<td>Res</td>
<td>6</td>
</tr>
<tr>
<td>Horse</td>
<td>High&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1:32</td>
<td>Res</td>
<td>6</td>
</tr>
<tr>
<td>Cow</td>
<td>High&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1:32</td>
<td>Res</td>
<td>6</td>
</tr>
<tr>
<td>Man</td>
<td>High&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1:32</td>
<td>Res</td>
<td>6</td>
</tr>
<tr>
<td>Resistant sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune g. pig</td>
<td>10⁶.⁸</td>
<td>1:4</td>
<td>V, Susc</td>
<td>24</td>
</tr>
<tr>
<td>Rat, NIH black</td>
<td>10⁶.⁸</td>
<td>1:4</td>
<td>V, Susc</td>
<td>17</td>
</tr>
<tr>
<td>Rat, Fischer 344</td>
<td>10⁶.⁸</td>
<td>1:4</td>
<td>V, Susc</td>
<td>24</td>
</tr>
<tr>
<td>Dog</td>
<td>1:0</td>
<td>1:0</td>
<td>Susc</td>
<td>33</td>
</tr>
<tr>
<td>Swine</td>
<td>1:0</td>
<td>1:0</td>
<td>Susc</td>
<td>9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Except as noted, from Young, Zelle, and Lincoln (10).<br><sup>b</sup>Albrink and Goodlow (1).<br><sup>c</sup>Est. from Young et al. (10) that rabbits and hamsters are two times as susceptible as guinea pigs.<br><sup>d</sup>Trnka et al. (Rosenwald et al., ms in preparation).<br><sup>e</sup>Estimate based on frequent mention of “large” number of organisms in blood taken at death.<br><sup>f</sup>Unpublished data.<br><sup>g</sup>Est. from Young et al. (10) four dogs given 1 × 10⁶ showed febrile reaction; one challenged at 1 × 10⁶ died.<br><sup>h</sup>Est. from Young et al. (10) resistance comparable to rat and dog.
susceptible group as regards dose required to establish anthrax, the terminal number of organisms is high, inhibition of phagocytes by toxin is great, it requires a high unitage of toxin to kill by intravenous injection and intracellular germination of spores in phagocytes is consistently different from the same parameters in the resistant group. This, in our opinion, is not a coincidence but an indication of general interrelationship of host-parasite interaction. The whole problem of extrapolation of disease response from experimental animals to man is so complex and difficult that we think it inappropriate to do more than suggest that the apparent relationship is real and that more work needs to be done to explore this model.

SEROLOGICAL SURVEY OF FIELD POPULATIONS

Of all the contagious diseases surveyed in the field, we know of no survey considering anthrax. This situation may be true because few scientific workers are interested in anthrax, and it is debatable whether a good serology system has been developed. Perhaps it is tacitly assumed that all anthrax infections are lethal, an assumption that does not seem reasonable considering the uncertainty of biological responses, and the prevalence of lowly virulent field strains and resistant species. In addition, more than one cell of Bacillus anthracis is required to cause infection, and cases of recovery have been reported. In South Africa, Sterne (9) reports that if blood smears were taken of every animal that died in the area surveyed, only 25% of anthrax deaths were diagnosed and reported. Dordevic (3), reporting on anthrax in man and animals in Yugoslavia, states that official data do not cover all the cases of anthrax, and that the number of cases are easily double those reported. It is also recognized that for political and economic reasons a country may not report anthrax, although it may occur at a significant rate.

A field survey would give much valuable information on incipient or controlled infections versus the observed or diagnosed ones. By obtaining both qualitative and quantitative information on antigens, such a survey would characterize Bacillus anthracis to a far greater degree than has yet been done. Certainly, such characterization would establish a) whether a strain specialization for bovine, goats, and the like, does or does not exist, and b) the prevalence of strains able to overcome the PA type immunization (2).

DISCUSSION

The translation of disease models from experimental hosts to man or his domesticated valuable animals might well be considered one of the most challenging and difficult problems for medical researchers. With a "lethal" disease, such as anthrax, the problems are greatly increased over those of a nonlethal one. Our comments have been made not to urge or deny specifically the value of a field test or survey but rather to note that a relationship exists among experimental species which affects our view on the epidemiology and treatment of anthrax. Where quantitative data are available, there is reasonable support of this hypothesis; however, too little is known about man and the domesticated animals for the suggested model to be broadly evaluated. In discussing this subject we had hoped to show the type of quantitative and qualitative data needed to more completely evaluate field anthrax, and thereby accumulate such information so that an evaluation can reasonably be made of how the model discussed in this paper applied to wild species endemically exposed to anthrax, or to man or his domesticated species.

SUMMARY

Data are presented to support the hypothesis that animals resistant to the establishment of anthrax are susceptible to its toxin, the former shown by dose of organisms and the latter by challenge with sterile toxin, and by the number of organisms and units of toxin per milliliter of terminal blood. The variables discussed are dose, doubling rate in the blood, terminal number of organism per milliliter of blood, units of toxin per milliliter of terminal blood, inhibition of phagocytosis by toxin, spore germination within the phagocyte, quantitative phagocytosis in vitro, and lysis of phagocytes in vitro. The need for quantitative information from field cases of anthrax is emphasized for its usefulness as research information per se and to more completely understand field anthrax. In addition, the information obtainable by a field serological survey and use of such information is discussed.

REFERENCES

Treatment of anthrax

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PROPHYLAXIS—ACTIVE IMMUNIZATION

Following the reports of Wright et al. (2, 15–19) on the successful development of an anthrax vaccine, a field study was set up in 1955 to determine the protective value of this antigen in a controlled industrial population chronically exposed to anthrax bacilli. Four plants that manufactured interlining for coats from imported goat hair as the raw material cooperated in this study. The results were reported by Brachman et al. (4). The heavy background contamination of these plants was established in a number of surveys (3, 6). The incidence of anthrax in these four plants, prior to initiation of the study, is shown in Table 1. The incidence is based on officially reported cases; actually it was probably higher in some of the plants.

Patients who had recovered from anthrax were excluded from the program. Those who volunteered were divided into two equal groups according to age, length of employment, and department in which they worked. One group received a vaccine supplied by Dr. Wright while the other got a placebo of 0.1% alum. None of the participants had either a choice or knowledge of the type of material that was injected. The dose scheduled for both groups was as follows: an initial series of three subcutaneous injections of 0.5 cc each given at intervals of 2 weeks, followed by a booster dose of 0.5 cc given every 6 months for three doses, then 0.5 cc given annually. “Complete” inoculation includes those employees who received all the injections as per schedule. “Incomplete” includes inoculated employees who missed one or more of the injections, whether vaccine or placebo. The number of participating employees is shown in Table 2.

The total eligible population at the initiation of the program was 1,249 people with 47% working in high-risk areas and 53% working in low-risk areas. Both the placebo and vaccine groups were pretty well equalized in these two areas.

Mill A had the largest group of refusals and it was at this mill that an epidemic occurred in 1957 with five cases of pulmonary anthrax. The refusals were made on a personal basis—dislike or fear of injections, poor health and the like; in plant M, in the horsehair section of the weaving department, only 25% received inoculation. The three who were injected were male loom fixers; the nine who refused were all women—average age 63 years, had worked in the same area for an average of 11.7 years, and had never had a case of anthrax. Yet the sampling program of this plant done a few months earlier (Jan. 1955) showed that 58.2% of surface samples from this department were positive for anthrax bacilli.

We could not adhere to the booster schedule and strict placebo control as outlined because an outbreak of inhalation anthrax in mill A in 1957, midway between the completed initial series and the due date of the first booster dose, forced cancellation of the program at this plant. Its entire personnel was then actively immunized with the vaccine. In plant S, the schedule was disrupted in the 3rd year of operation of the program because of relocation of the spinning and weaving departments to a subsidiary plant in the South. There occurred a gradual decline of 13–20% in the follow-up population after each successive booster inoculation. The program was officially terminated at the end of 4 years when enough cases of anthrax had accumulated as a result of “natural” occupational exposure to make the analysis of results statistically significant.

In 1961, at mill S where 85 employees were still working, a pulmonary anthrax infection occurred in an unimmunized office secretary; thereafter all personnel was actively vaccinated. At this plant, in a period of 10 years (1955–1965) the employees had received from 4 to 10 injections of vaccine.

During the period of evaluation, 26 cases of anthrax were reported. At mill A, nine cases occurred in a 10-week period, indicating an actual epidemic (1, 5, 14). Five of these cases were of the inhalation or pulmonary variety. The remaining 21 were cases of cutaneous anthrax, which in mills M, P, and S occurred throughout the entire period of evaluation. These cases are summarized in Table 3.

Three cases of anthrax occurred in the vaccinated population. The remaining 23 developed among the placebo or un inoculated control population.

Of the three who had received the vaccine, one patient AC (mill S) received two doses, and developed anthrax of the hand a day or two prior to the scheduled third or final dose of the initial series. Since she had not completed the basic course of immunization, this anthrax infection cannot be charged as a failure of the vaccine.

The second patient MS (mill S) had received the initial three injections of vaccine but developed anthrax
of the leg about 5 months later, just before the first booster dose was due.

The third patient \( JW \) (mill P) developed anthrax of the forearm 13 months after the initial series. He had never received the first booster dose of vaccine. The significance of these two cases will be discussed later.

Of the placebo patients, two were “incomplete.” \( SW \) (mill P) developed anthrax of the arm 8 months after the last inoculation of her initial series and \( RP \) (mill A) developed anthrax of the wrist 3 months after the last inoculation of an “incomplete” initial series. Fifteen additional cases of anthrax occurred at all stages of complete placebo inoculation in decreasing numbers, reflecting the decreasing population available for successive injections. The other six cases of anthrax occurred in immunized personnel.

Statistical analysis of these data was performed by Dr. R. E. Scifling, Chief of the Statistics Section, Epidemiology Branch, Communicable Disease Center. It included high- and low-risk groups with “complete” inoculations only. Using the attack rate in the placebo group calculated per 1,000 person-months, the total expected cases for the entire vaccinated group was calculated as 13.32. Since only one case occurred, the effectiveness of the vaccine was found to be 92.5%, with an estimate of 65% as a lower 95% confidence limit for effectiveness of the vaccine.

A similar analysis of 81 individuals in the study population who had previously had anthrax was made. Using the attack rates observed for the placebo group, 5-73 cases would have been expected in this group; however, no cases were observed, which suggests that some protection resulted from the previous anthrax infection.

Since the program was instituted in 1955 at plant S, 13 cases of anthrax have been observed to date (Table 4). Two of these occurred in 1941, both in uninoculated individuals. One patient died from an unrecognized pulmonary anthrax infection; the other was a cutaneous lesion.

In the dosage schedule used, it would appear that three doses are necessary for the initial inoculation. The protection that ensues may not last more than a few months as exemplified by one of our patients and two at Camp Detrick who developed anthrax 5 months after the initial series were completed (H. N. Glassman, personal communication). However, it is quite possible that a longer time interval such as 8-10 weeks between the first two doses of vaccine may give better protection than the present schedule of three doses given every 2 weeks.

Our data suggest that in order to secure adequate protection, one or perhaps two booster doses should be given at 3-month intervals after the initial course of immunization. Thereafter, our data are not conclusive. From 1957 to 1961, 66 of the patients at plant S received no further injections, yet no cases of anthrax were recorded for 1958, 1959, and 1960. Similarly no injections were given after August 30, 1962 until November 1, 1965, and again in this 3-year period no cases of anthrax were seen at this plant. This would suggest that once immunity has been established, booster doses may be spaced at 2- to 3-year intervals. The whole question of dosage schedule deserves further investigation.

It may be argued that other factors account for the disappearance of anthrax from plant S during these years besides vaccination.

1) Reduction in number of exposed population; some of the plant operations had been transferred to a subsidiary in the South and the number of personnel who had recovered from previous anthrax infection had shown a relative as well as absolute increase in numbers, especially in the high-risk areas.

2) The introduction in 1959 of clipped hair imported

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**Table 1. Incidence of anthrax in four mills prior to initiation of vaccination program**

<table>
<thead>
<tr>
<th>Mill</th>
<th>Average Total Employment</th>
<th>Cases of Anthrax, 1958 to Initiation of Study*</th>
<th>Cases/100 Mill Employees per Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>655</td>
<td>63</td>
<td>1.0</td>
</tr>
<tr>
<td>M</td>
<td>227</td>
<td>23</td>
<td>1.4</td>
</tr>
<tr>
<td>P</td>
<td>148</td>
<td>7</td>
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</tr>
<tr>
<td>S</td>
<td>300</td>
<td>38</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>1,390</td>
<td>130</td>
<td>1.2</td>
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**Table 2. Participation of employees in anthrax vaccine evaluation program**

<table>
<thead>
<tr>
<th>Mill</th>
<th>High Risk</th>
<th>Low Risk</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculated</td>
<td>Refusal</td>
<td>Total</td>
</tr>
<tr>
<td>Vac*</td>
<td>Plac†</td>
<td>Inc‡</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>59</td>
<td>60</td>
<td>11</td>
</tr>
<tr>
<td>M</td>
<td>42</td>
<td>49</td>
<td>15</td>
</tr>
<tr>
<td>P</td>
<td>19</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>S</td>
<td>89</td>
<td>95</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>209</td>
<td>226</td>
<td>65</td>
</tr>
</tbody>
</table>

| Inoculated | Refusal | Total |
| Vac* | Plac† | Inc‡ |
| A    | 90    | 104  | 24   |
| M    | 31    | 42   | 4    |
| P    | 22    | 22   | 13   |
| S    | 27    | 20   | 10   |
| Total| 170   | 188  | 51   |

| Inoculated | Refusal | Total |
| Vac* | Plac† | Inc‡ |
| A    | 149   | 164  | 25   |
| M    | 73    | 91   | 12   |
| P    | 41    | 44   | 13   |
| S    | 116   | 115  | 216  |
| Total| 379   | 414  | 1,249 |

*Vaccinated. †Placebo. ‡Incomplete.
from Pakistan and Iran instead of pulled hair. The former is a much cleaner product.

3) Sterilization of hair before it leaves plant S for North Carolina as required by that state. This is a makeshift procedure of questionable value. That these factors are not significant in the assessment of the value of vaccination is attested by the fact that in 1941, two cases of anthrax occurred at plant S both in unimmunized personnel—one a fatal pulmonary infection case. Also, in 1963 a case of cutaneous anthrax occurred in the North Carolina subsidiary plant in a recently hired spinner, 1–2 days after she had received the first dose of vaccine. This is the only case of anthrax that occurred in this plant since 1957 when vaccination with Wright’s vaccine was made mandatory. This is particularly striking in view of the fact that the turnover in hired personnel at this plant is very high.

Although multiple injections were given, the incidence of local reactions was small and the latter was never severe except in an occasional individual. We encountered only one patient, a middle-aged woman office worker, who developed fever, malaise, abdominal cramps, and diarrhea after each booster injection. These systemic manifestations subsided overnight and were not accompanied by any undue local reaction.

### Table 3. Summary of cases of anthrax in the evaluation population

<table>
<thead>
<tr>
<th>Date of Onset</th>
<th>Name</th>
<th>Age</th>
<th>Mill</th>
<th>Dept</th>
<th>Length of Employment, yr</th>
<th>Vaccine Status</th>
<th>Site of Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-14-55</td>
<td>AC</td>
<td>24</td>
<td>S</td>
<td>Spinning</td>
<td>4.5</td>
<td>V-I</td>
<td>Hand</td>
</tr>
<tr>
<td>3-30-55</td>
<td>JK</td>
<td>36</td>
<td>S</td>
<td>Carding</td>
<td>4.0</td>
<td>P-C</td>
<td>Nose</td>
</tr>
<tr>
<td>5-19-55</td>
<td>GW</td>
<td>30</td>
<td>S</td>
<td>Spinning</td>
<td>4.0</td>
<td>P-C</td>
<td>Finger</td>
</tr>
<tr>
<td>5-27-55</td>
<td>VY</td>
<td>48</td>
<td>S</td>
<td>Spinning</td>
<td>10.0</td>
<td>P-C</td>
<td>Hand</td>
</tr>
<tr>
<td>9-4-55</td>
<td>MS</td>
<td>33</td>
<td>S</td>
<td>Spinning</td>
<td>9.5</td>
<td>V-C</td>
<td>Hand</td>
</tr>
<tr>
<td>11-1-55</td>
<td>ES</td>
<td>27</td>
<td>S</td>
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<td>P-C</td>
<td>Leg</td>
</tr>
<tr>
<td>11-11-55</td>
<td>MG</td>
<td>58</td>
<td>S</td>
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<td>P-C</td>
<td>Forearm</td>
</tr>
<tr>
<td>11-18-55</td>
<td>MV</td>
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<td>P-C</td>
<td>Cheek</td>
</tr>
<tr>
<td>1-31-55</td>
<td>MV</td>
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<td>S</td>
<td>Spinning</td>
<td>4.0</td>
<td>U</td>
<td>Finger</td>
</tr>
<tr>
<td>6-5-55</td>
<td>HK</td>
<td>38</td>
<td>S</td>
<td>Carding</td>
<td>6.0</td>
<td>P-C</td>
<td>Wrist</td>
</tr>
<tr>
<td>6-18-55</td>
<td>ES</td>
<td>43</td>
<td>S</td>
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<td>P-C</td>
<td>Forearm</td>
</tr>
<tr>
<td>8-16-56</td>
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<td>M</td>
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<td>Forearm</td>
</tr>
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<td>P-C</td>
<td>Forearm</td>
</tr>
<tr>
<td>2-15-57</td>
<td>N J</td>
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<td>S</td>
<td>Carding</td>
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<td>P-C</td>
<td>Neck</td>
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<tr>
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<td>P</td>
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<td>P-C</td>
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</tr>
<tr>
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<td>P-C</td>
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<td>A</td>
<td>Carding</td>
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<td>U</td>
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<td>M</td>
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</tr>
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<td>P-C</td>
<td>Finger</td>
</tr>
<tr>
<td>10-10-57</td>
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<td>A</td>
<td>Weaving</td>
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<td>U</td>
<td>Finger</td>
</tr>
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<td>P-I</td>
<td>Inhalation</td>
</tr>
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<td>10-30-57</td>
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</tr>
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</tr>
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<td>P-C</td>
<td>Finger</td>
</tr>
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<td>P</td>
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<td>V-I</td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* P—placebo, V—vaccine, U—uninoculated, C—complete, I—incomplete, ¶ known. † Months.

### Table 4. Summary of cases of anthrax in the evaluation population (mill S)

<table>
<thead>
<tr>
<th>Date of Onset</th>
<th>Name</th>
<th>Age</th>
<th>Mill</th>
<th>Dept</th>
<th>Length of Employment, yr</th>
<th>Vaccine Status</th>
<th>Site of Lesion</th>
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</thead>
<tbody>
<tr>
<td>3-14-55</td>
<td>AC</td>
<td>24</td>
<td>S</td>
<td>Spinning</td>
<td>4.5</td>
<td>V-I</td>
<td>Hand</td>
</tr>
<tr>
<td>3-30-55</td>
<td>JK</td>
<td>36</td>
<td>S</td>
<td>Carding</td>
<td>4.0</td>
<td>P-C</td>
<td>Nose</td>
</tr>
<tr>
<td>5-19-55</td>
<td>GW</td>
<td>30</td>
<td>S</td>
<td>Spinning</td>
<td>4.0</td>
<td>P-C</td>
<td>Finger</td>
</tr>
<tr>
<td>5-27-55</td>
<td>VY</td>
<td>48</td>
<td>S</td>
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<td>16.0</td>
<td>P-C</td>
<td>Hand</td>
</tr>
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<td>9-4-55</td>
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<td>V-C</td>
<td>Hand</td>
</tr>
<tr>
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<td>P-C</td>
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</tr>
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<td>P-C</td>
<td>Cheek</td>
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<td>1-31-55</td>
<td>MV</td>
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<td>S</td>
<td>Spinning</td>
<td>4.0</td>
<td>U</td>
<td>Finger</td>
</tr>
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<td>6-5-55</td>
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<td>S</td>
<td>Carding</td>
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<td>P-C</td>
<td>Wrist</td>
</tr>
<tr>
<td>6-18-55</td>
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<td>Forearm</td>
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<td>18</td>
<td>U</td>
<td>Inhalation</td>
</tr>
<tr>
<td>12-12-56</td>
<td>AC</td>
<td>24</td>
<td>S</td>
<td>Spinning</td>
<td>4.5</td>
<td>V-I</td>
<td>Hand</td>
</tr>
<tr>
<td>12-17-56</td>
<td>JC</td>
<td>18</td>
<td>S</td>
<td>Finishing</td>
<td>1.0</td>
<td>U</td>
<td>Forearm</td>
</tr>
</tbody>
</table>

* P—placebo, V—vaccine, U—uninoculated, C—complete, I—incomplete. † Months.

### Treatment

**Cutaneous anthrax.** My experience with the treatment of anthrax spans 30 years (Tables 5 and 6) and covers the entire spectrum of therapeutic agents, from the early use in 1933 of small doses of antianthrax horse serum intravenously and around the “pustule,” to massive doses 1,000 cc of antianthrax horse serum given in a single intravenous injection, to repeated large doses of neosarophenamine, to the sulfonamides, and lastly to penicillin and the broad-spectrum antibiotics: tetracycline, erythromycin, chlorotetracycline, oxytetracycline and Chloromycetin (7–12).

The results of treatment of cutaneous anthrax are excellent, especially if the disease is promptly recognized and diagnosis established early by smear and culture.

The incubation period may last 12 hr to 5 days, with an average of 3 days. A history of trauma is frequently elicited but is not essential. In many cases, the injury may be so trivial as to escape notice.

First, let me emphasize that therapy of anthrax is exclusively systemic in nature. The local lesion must not be approached with a surgical knife. It should neither be incised nor excised; the bacteriologic examination should involve gentle stroking of the pustule with a moist, sterile applicator. The lesion should be covered at all times with a heavy dressing to be removed by the attending physician, as needed. He, alone, must assume responsibility for its proper disposal.

A word about prophylactic therapy. After injury has occurred in an anthrax-contaminated environment, the question of preventing cutaneous anthrax comes up. I have used both penicillin and the broad-spectrum antibiotics for this purpose, with uncertain results.

Certainly, the recovery of anthrax bacilli from the skin does not signify that infection will occur; hence it is
difficult, if not impossible, to credit any given antibiotic therapy with prophylactic value if a pustule fails to develop. However, the converse is true. Patient 100, in my series, with severe injuries of the right hand and forearm that involved multiple fractures and third-degree burns, received large doses of penicillin following the accident, but on the 5th day of hospitalization developed the most extensive anthrax eschar that I have seen; it covered the entire external surface of the hand and forearm. Recovery occurred after additional intensive treatment with penicillin, oxytetracycline, and Chloromycetin.

For the record, it must be stated that in an occasional patient cutaneous anthrax may be so mild in course as to require no treatment. I have encountered two such cases.

Regardless of the therapeutic agent, its adequacy is measured clinically by a simple yardstick—control of the spreading edema. After this is accomplished, the painless swelling recedes in 24-48 hr. Shortly thereafter, the pain and tenderness over the regional lymph nodes subside, and the systemic manifestations, fever, tachycardia, and malaise, disappear. The local lesion, that is, the anthrax pustule, is not directly affected by therapy. It evolves through the same course as the untreated lesion. Vesiculation, eschar formation with spread into the periphery, separation, and finally, granulation of the ulcerated area take about 2 weeks. Of interest is the observation that small, palpable regional lymph nodes may persist for weeks after the skin lesion has healed and the patient is otherwise well. This is no indication for continued treatment.

At present, penicillin is the antibiotic of choice in the treatment of anthrax. The dosage and method of administration, oral or parenteral, will depend on the individual case. In mild infections with little edema, oral Pen-Vee, 250 mg every 6 hr will suffice. In more severe cases, procaine penicillin 600,000-1,200,000 units may be given intramuscularly every 12 hr for a day or two, when spread of the edema will be stopped. Aqueous penicillin, in doses of 1,000,000 units every 6 hr should be given intramuscularly to patients who are seen late in the disease with extensive edema, or when the lesion is located on the head or neck where respiratory embarrassment may become a severe complication.

After adequate initial doses of penicillin, the edema is controlled in 24-48 hr; thereafter, the daily dose of antibiotic is reduced, and in 3 or 4 days treatment may be stopped. Usually the smear and culture become negative for Bacillus anthracis in 24-48 hr after injection of penicillin. However, in one patient, 101, the organism was recovered 60 hr after the treatment was begun and patient had received a total of 3,300,000 units of penicillin in oil.

In order to avoid multiple injections, possible sensitization reactions, and hospitalization, the broad-spectrum antibiotics were tried in the treatment of anthrax and found to be quite effective.

The results of in vitro sensitivity tests are shown in Table 7. In vitro, the B. anthracis appeared to be most sensitive to Terramycin, then to Aureomycin, penicillin, Chloromycetin, and streptomycin, in the order given. In vivo, Aureomycin, tetracycline, Terramycin, erythromycin, chloramphenicol all work well, but their effect is a little slower than with penicillin, as judged by the interval of time that elapses before the spread of edema is controlled, and before the smear and culture from lesion become negative (2-5 days).

<table>
<thead>
<tr>
<th>TABLE 6. Distribution of cutaneous anthrax cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
</tr>
<tr>
<td>Department of local mill</td>
</tr>
<tr>
<td>Carding</td>
</tr>
<tr>
<td>Spinning</td>
</tr>
<tr>
<td>Drawing</td>
</tr>
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<td>Finishing</td>
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<td>Doffing</td>
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<td>Burling</td>
</tr>
<tr>
<td>Warehouse</td>
</tr>
<tr>
<td>Sweeper</td>
</tr>
<tr>
<td>Machinist</td>
</tr>
<tr>
<td>Village resident</td>
</tr>
<tr>
<td>Children</td>
</tr>
<tr>
<td>Philadelphia subsidiary mill</td>
</tr>
<tr>
<td>Truckers</td>
</tr>
<tr>
<td>New Jersey farmer</td>
</tr>
<tr>
<td>Local mill (pulmonary anthrax)</td>
</tr>
<tr>
<td>Office secretary</td>
</tr>
<tr>
<td>Carder</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
TABLE 7. Bacterial sensitivity tests*  

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Penicillin μg</th>
<th>Aureomycin μg</th>
<th>Terramycin μg</th>
<th>Chloramphenicol μg</th>
<th>Streptomycin μg</th>
<th>Neomycin μg</th>
<th>Polymyxin μg</th>
<th>Tetracycline μg</th>
<th>Serial Dilutions</th>
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<tr>
<td>84</td>
<td>&lt;0.1</td>
<td>&lt;0.1*</td>
<td>3.12</td>
<td>3.12</td>
<td>5.0</td>
<td>&gt;25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>pi 0.1</td>
<td>pi 0.1</td>
<td>3.12</td>
<td>pi 3.12</td>
<td>5.0</td>
<td>&gt;25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>ci 1.0</td>
<td>ci 1.0</td>
<td>3.12</td>
<td>pi 3.12</td>
<td>5.0</td>
<td>&gt;25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>3.12</td>
<td>pi 3.12</td>
<td>5.0</td>
<td>&gt;25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>pi 1.0</td>
<td>pi 1.0</td>
<td>3.12</td>
<td>pi 12.50</td>
<td>5.0</td>
<td>&gt;25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>ci 5.0</td>
<td>ci 5.0</td>
<td>3.12</td>
<td>pi 12.50</td>
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<td>&gt;25.0</td>
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<tr>
<td>90</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>3.12</td>
<td>pi 12.50</td>
<td>5.0</td>
<td>&gt;25.0</td>
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</tr>
<tr>
<td>91</td>
<td>pi 0.1</td>
<td>&lt;0.1</td>
<td>3.12</td>
<td>pi 12.50</td>
<td>5.0</td>
<td>&gt;25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>92</td>
<td>ci 1.0</td>
<td>&lt;0.1</td>
<td>3.12</td>
<td>pi 12.50</td>
<td>5.0</td>
<td>&gt;25.0</td>
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</tr>
<tr>
<td>93</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>3.12</td>
<td>pi 12.50</td>
<td>5.0</td>
<td>&gt;25.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>3.12</td>
<td>pi 12.50</td>
<td>5.0</td>
<td>&gt;25.0</td>
<td></td>
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</tr>
<tr>
<td>110</td>
<td>1 U/ml</td>
<td>4 μg/ml</td>
<td>6 μg/ml</td>
<td>15 μg/ml</td>
<td>5 μg/ml</td>
<td></td>
<td></td>
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<tr>
<td>111</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
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<td></td>
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<tr>
<td>112</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>113</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* pi = Partial inhibition, ci = complete inhibition. † Performed in the Laboratory of the Division of Infectious Diseases of the Hospital of the University of Pennsylvania through the courtesy of Drs. W. P. Boger and E. L. Foltz.

Individual response to each antibiotic varies, and in a given patient one may have to switch antibiotics if the response is not prompt or satisfactory. The dose by mouth is 500 mg every 4–6 hr for 2–3 days, then at decreased or lengthened intervals until edema subsides. The usual gastrointestinal reactions to the broad-spectrum antibiotics were encountered in our patients.

Inhalation anthrax. Effective treatment of inhalation anthrax and its secondary localization, meningitis, is possible provided the diagnosis is made early enough. One of the five patients of pulmonary anthrax, in the Manchester epidemic, survived after treatment with penicillin. The two cases that occurred at plant S died before the etiologic diagnosis was established at autopsy.

A high index of suspicion is essential, and treatment should be prompt and energetic. Penicillin should be given in the same dosage as for bacterial endocarditis, a continuous intravenous drip of 20,000,000 units in 24 hr is preferable. Streptomycin, 1–2 g/day given im, may have a synergistic effect when combined with penicillin, as suggested by the experimental cures of septiemic anthrax in rhesus monkeys obtained by Lincoln et al. (13). If successful, treatment should be continued for at least 14 days to prevent reinfection bacteremia.

Death is the result of anthrax toxemia, and ideally treatment should include a specific antitoxic serum. Unfortunately, this is not available. Supportive treatment, aimed at maintaining circulation and fluid-electrolyte balance requires special attention.

CONCLUSIONS

1) Active immunization against anthrax by means of the Wright antigen is successful and should be used in chronically exposed industrial populations and by technicians and veterinarians who handle anthrax-contaminated material. The dosage schedule used by Brachman et al. (4) deserves further investigation.

2) Treatment of cutaneous anthrax by means of penicillin or broad-spectrum antibiotics results in cure if instituted early. Massive doses should be used in late cases.

3) Pulmonary anthrax should respond to combined penicillin-streptomycin therapy if treatment is instituted early. A high index of diagnostic suspicion is essential to secure cure.

REFERENCES

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General summary of the anthrax conference

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I wish to thank the organizers of this Conference for permitting me to learn so much about anthrax. It has been a rewarding 2 days. Quite a few of the participants have described problems worthy of investigation. I thought I might list some of these, with comments where relevant, and then add some ideas of my own.

Dr. Gerhardt had interesting data on the ability of anthrax spores of an avirulent strain to germinate freely within alveolar macrophages with subsequent killing of the phagocytic cells. This work should be extended to include periods of time longer than 4 hr and with other types of phagocytes included. Indeed, the role of the reticuloendothelial system in the animal's response to anthrax should be clarified.

The work with anthrax toxins described by Drs. Smith, Fish, Ward, and their associates is putting the pathogenesis of the disease in clearer perspective. Quite obviously the exotoxins produced by the bacteria cause the disease. Purification of these materials should certainly continue. The importance, if any, of fractions isolated but not yet identified by Dr. Ward and her group should be elucidated. Moreover, the immune response to the different toxins, including the protective antigen, merits study in depth. Possibly related to the toxins and the substances responsible for pathogenesis are Dr. Williams' observations with protocatechuic acid and its relationship to epinephrine. I am sure this lead interests him too much for him to fail to pursue it.

Dr. Stoner's suggestion that anthrax toxins provide a tool for physiologists interested in vascular problems is worthy of emphasis. These substances are almost unique in producing an edema that is slow in onset and offer an experimental model for detailed analysis. Dr. Stoner also mentioned that the high susceptibility of the Fischer rat might be studied from a genetic point of view. Susceptibility and resistance to certain enteric and viral diseases has been analyzed genetically in mice by such workers as Webster and Gowen, and it should be of interest to extend this to anthrax as well.

The work Dr. Walker and his associates have been doing with the biochemistry and physiology of response to anthrax toxins and to anthrax infections is one of especial interest to me. If man is to ultimately understand infectious diseases, he must do so at this level. Dr. Walker has made significant progress. It looks as if the final event preceding death from toxins is respiratory paralysis, at least as judged by the absence of discharges along the phrenic nerve. Whether or not the central nervous system is the primary site of action of the toxin requires further study. The observation that toxin injected directly into the cerebrospinal fluid of monkeys precipitates death in 12 min rather than in 18 hr following intravenous administration is interesting but does not necessarily prove that leakage into the brain normally occurs. That such monkeys can be kept alive by means of artificial respiration during a comparatively few critical moments indicates a rapid destruction or elimination of the toxin. When death does occur in these animals the critical events are highly transitory. Similar conclusions would have to be drawn on the basis of the ability of isoproterenol to protect against toxic death. Whatever encouragement Dr. Walker and his associates need to continue with their studies should certainly be given.

Dr. Bonventre had several interesting suggestions to make. The labeling of toxins or antitoxins for studies of localization within the host may make it possible to identify target areas. His suggestion to relate permeability changes to injections of edema factor is meritorious. These studies might profitably be extended to include perfusion experiments with lungs and other organs as possibly a more refined method for detecting changes in permeability. The in vitro production of lesions in tissue cultures might also be related to altered cell permeability.

Dr. Smith emphasized the importance of combining the three toxic fractions, Factors I, II, and III (or edema factor, protective antigen, and lethal factor) to see if their necrotizing effect is greater than that obtained with each alone. Dr. Smith also believed it feasible and important to follow changes in capillary permeability as possibly one of the primary effects of anthrax toxins and the one responsible for the edema.

Mr. Rhian suggested that phagocytic cells are capable of removing anthrax toxin under in vivo conditions. This could be tested experimentally by incubating toxin in the presence of different types of phagocytes prior to injection into susceptible hosts. Strain differences as well as species differences in susceptibility have been emphasized throughout the Conference and should be studied whenever possible. Possibly phagocytes vary in ability to remove toxin or to detoxify it.

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Dr. Lincoln presented elegant data on organ counts of anthrax bacilli at different times postinfection, along with the bacteremic level of organisms at the time of death, and related this to the susceptibility of the animal to toxin and to the specific susceptibility of the species. These are interesting observations and should be extended to include counts of total pathogens to see the extent to which susceptible and resistant species differ.

I have been struck by the absence of comment on the role temperature might play in response to anthrax infection or anthrax toxin administration. I would presume that animals remain essentially normothermic throughout the incubation period of the disease but it would be of interest to follow the change in body temperature of different species, particularly under different environmental temperatures, to see what role, if any, thermoregulation plays in the host. It would also be of interest to see how reticuloendothelial blockade might alter susceptibility to the toxin, including the effect it might have on body temperature regulation.

Dr. Walker and his group have evidence that carbohydrate metabolism in anthrax-infected or toxin-poisoned animals is impaired. This is a common response to various types of stress, not only that produced by infection but by hemorrhage and tourniquet shock as well. It may be that the lability in reserves of body carbohydrates is related to impaired gluconeogenesis. Two enzymes in the liver, phosphoenolpyruvic carboxykinase and pyruvate carboxylase, each catalyzing crucial steps in the synthesis of sugars from monosaccharides in precursors, have a half-life of approximately 2 hr, are hormonally inducible, and hence are subject to rapid changes. It could be that these enzymes are the ones most easily affected under these conditions.

The homeostatic regulation in animals and man subjected to various types of infection or trauma may be altered. Homeostasis should not be conceived as a process by which true constancy of the internal environment is maintained but rather one that is controlled within regularly fluctuating limits. It is perhaps the fluctuations that play an important role in regulatory mechanisms, and in animals subjected to stress the adrenal cortex is essential to its ability to adjust and survive. Most likely, one of the phenomena involved in such an adjustment is enzymic regulation. At the present time, quite a few liver enzymes are known to be inducible by adrenal cortical hormones and the manner in which an infectious disease may alter enzymic regulation should be investigated. On the basis of our own observations, this seems to be one of the key mechanisms involved in endotoxin poisoning, and it might well prove valuable to examine this effect in anthrax. Many responses are known to be mediated and it looks as if this may be true for the response to the adrenal cortical hormones. A liver slice, for example, under in vitro conditions, responds poorly, if at all, to cortisone. In addition, slices fail to respond in a measurable way to endotoxin, despite the fact that the liver seems to be a primary target for both substances. It would be of value, therefore, to approach a study of anthrax pathogenesis through organ culture or tissue slice experiments to see if a primary lesion can be identified.

In closing, I would like to point out that anthrax would serve admirably as a model infection, especially if the toxins could be made available to investigators. Greater understanding of their nature and mode of action would likely be forthcoming within a short period of time. It is a disease, however, that most laboratories are reluctant to study because of the resistance of the spores and the hazards involved in its handling.

Let me say once again that it has been a pleasure to be associated with this group of people. I have enjoyed the 2 days very much indeed.
Problems for future studies of anthrax

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1) More accurate reporting of incidence of anthrax in animals and man on a worldwide basis.

2) Development of the natural history of Bacillus anthracis in nature as related to its survival and possible multiplication in soil, resistant animals, birds, insects, and on plants.

3) Improved knowledge as to portal of entry of B. anthracis or its spores into animals, particularly wild animals.

4) Further study of mechanisms responsible for germination of spores in vivo.

5) Determination of factors affecting the destruction of spores and vegetative cells of avirulent and virulent organisms by various phagocytic cells of different animal species.

6) A continuation of work in characterization of soluble factors produced by the bacterium in vivo or in vitro in terms of their biological, chemical, and physical properties. Emphasis needs to be placed on the antigenic properties of such substances as well as their ability to produce various adverse actions in the host.

7) There is a need for a common, even if temporary, nomenclature to be used in referring to the biologically active fractions so far isolated as well as those to be isolated.

8) There is need for additional information on the gross and histological pathology of anthrax including the changes to be noted with the aid of the electron microscope. Such future studies of the pathology of the disease should include animals varying in their resistance to the disease.

9) Further studies on the pathophysiology and pathobiology are indicated to capitalize on the good progress made to date and ultimately to understand the cause of death in this disease. Such information could then serve as a model for similar studies in other infectious diseases, a sorely neglected area.

10) The observation that animals at an elevation of 6,000 feet or greater above sea level rarely contract the disease requires confirmation and explanation. Do such conditions and the related cooler ambient temperature affect the resistance of the host or the number of bacteria to which it is exposed?

11) An extension is warranted of quantitative bacteriological and host defense-parasite interactions in animals of varying innate host resistance. It is hoped that from such studies information will be developed making it possible to define in a more meaningful manner host resistance and parasite virulence. Possibly such studies could even be extended to determining the virulence of a pathogen for man in terms of the effect of isolated host-defense mechanisms on a pathogen in vitro.

12) Determine the feasibility of actively immunizing wild animals with appropriately attenuated strains of B. anthracis delivered by aerosols.

13) Improve the active immunization of man with selected and prepared “toxoids” or strains of attenuated living bacteria to be developed.

14) Develop adequate means for disinfecting contaminated soils and water holes frequented by wild or domestic animals.

15) Improve the treatment of anthrax by developing means for neutralizing toxin circulating in the blood or by developing procedures to offset the pathophysiology resulting from toxemia.