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The Experimental Analysis of Tuberculous Infections

By RENÉ J. DUBOS, New York[1]

For almost a century, the morbidity and mortality rates of tuberculosis have been declining at an almost constant rate in North America, as well as in many European and other countries. Nevertheless, it has been estimated that in the United States approximately 50 per cent of the total population become infected with tubercle bacilli, although only a very small percentage show signs of clinical disease. Nevertheless, tuberculosis remains the greatest single cause of death in the 15–35 age group. Both morbidity and mortality rates remain at an extremely high level and even appear to be increasing at an alarming rate in a large portion of the rest of the world—in practically all of Asia, for example. Death rates of several hundred per 100,000 population are common in all Latin-American cities, where a full-fledged epidemic state appears to be prevailing, with an enormous percentage of the population suffering from active tuberculosis.

Many hypotheses have been advanced to account for this complex epidemiological picture; the most widely held views can be illustrated by a brief statement of the relation of tuberculosis to the process of urbanization. In Europe and North America, tuberculosis reached a high level of severity early in the nineteenth century, concomitantly with the beginning of the Industrial Revolution. The decline in morbidity and mortality began in the second half of the century long before the antituberculosis campaigns in their various forms had gained their momentum, and it occurred without the benefit of any purposeful prophylactic or therapeutic measures. The early decades of industrialization had been characterized by migration of labor into the cities, long working hours, poor nutrition, crowded housing, etc. All these factors had probably contributed to increase the rate of infection and the severity of the disease in a population of rural origin and exhibiting a high degree of susceptibility. After a few decades industrial prosperity was reflected in better living conditions for the workers; it is also possible that in consequence of the rapid spread and severity of tuberculosis in the early part of the century there may have occurred a selective elimination of the most susceptible members of the population, as well as a certain amount of immunization of the survivors as a result of exposure to small infective doses. A combination of these different factors might account for the decline in tuberculosis which became obvious sometime after 1850. There are indications that we are witnessing at the present time in Latin America the effects on tuberculosis of the first phase of industrialization. In this case again, the change from a pastoral to an industrial type of civilization results in the exposure of a rural population to increased opportunities for infection under unfavorable economic circumstances.

Although the foregoing analysis of the epidemiology of tuberculosis appears plausible, it rests on a number of ill-defined and unproven assumptions. For example, if it is true that poor nutrition increases susceptibility to tuberculosis—and this is surmised only from indirect evidence—it would be important to define which types of nutritional deficiencies are responsible for the phenomenon, and through what mechanism they affect the course of the disease. That severe epidemic periods result in a weeding out of the most resistant stock is an interesting epidemiological hypothesis; unfortunately, no technique is available to establish its validity, since we do not know how to evaluate natural susceptibility and resistance. It may also be true that, as the bacillus becomes established in a given population, opportunities are given for low grade infections which determine a certain degree of immunity without resulting in clinical disease; this, however, is difficult to prove for lack of sufficient knowledge concerning the mechanism of immunity to tuberculosis, or the measurement of its level.

Awareness of our ignorance of the factors which control the epidemiology of tuberculosis is not only of academic interest. The sustained fall in morbidity and mortality of the disease has led many to envisage its complete eradication from the United States within a few generations. This hope implies the tacit assumption that the factors—whatever they may be—which were responsible for the decrease of tuberculosis in the past will keep on operating in the future: most programs of control and eradication are based on this assumption. Unfortunately, as pointed out, there are many gaps in our understanding of the fac-

[1] The observations and views reported in this article are the result of a program of investigation carried out at the Rockefeller Institute for Medical Research, New York, in collaboration with Drs BERNARD D. DAVIS, GARDNER MIDDLEBROOK and CYNTHIA PIERCE.

tors which affect the natural history of the disease; it appears unjustified, therefore, to extrapolate past experience into the future and to feel confident that methods based on present day knowledge will be adequate to meet the problems arising in a different epidemiological constellation.

In general the experimental studies of tuberculosis have been based on attempts to reproduce in experimental animals a disease picture as similar as possible to that which occurs in man. It is not necessary to review here the great achievements which have come from this experimental approach and which have contributed so much to our understanding of the pathogenesis and histopathology of the disease. We have considered, on the other hand, that additional knowledge might result from a more analytical approach aiming at the separation of the different components of the host-parasite complex and not at their simultaneous reproduction in one single experimental disease. The separate aspects of experimental tuberculosis which are under consideration in our laboratory can be grouped as follows: the biological and chemical properties of the tubercle bacillus, and in particular those attributes which determine pathogenicity; the factors—hereditary and environmental—which condition the response of the host to infection; the different immunological reactions elicited by the different components of the bacterial cell and which determine the immune and allergic states; the possible utilization of immune protective mechanisms and of chemotherapeutic agents to affect favorably the course of infection. It has long been obvious that the experimental analysis of these different aspects of the host-parasite relationship is greatly handicapped by the technical difficulties involved in the cultivation of the tubercle bacillus *in vitro* and by limitations of the classical methods of animal experimentation with guinea pigs. It appears worth-while, therefore, to present in the following pages a number of observations which may be useful for the development of more satisfactory experimental methods.

Factors effecting the growth of tubercle bacilli in vitro

Although virulent tubercle bacilli can yield abundant growth when seeded on the surface of a variety of simple synthetic media, they usually fail to multiply unless the inoculum contains a considerable number of living cells. Even in the presence of the growth-promoting substances added in the form of organic materials (serum, egg yolk, potato extract, etc.) growth develops very slowly, if at all, when only a few cells are used for inoculation. These characteristics render difficult the application to tubercle bacilli of quantitative bacteriological methods based on enumeration of living cells by plating or dilution techniques; they delay and at times prevent bacteriological diagnosis of tuberculosis; they hinder investigations concerned with pathogenesis, immunity and chemotherapy.

In addition to these difficulties there are others, less obvious but equally important, which result from the heterogeneity of fully grown cultures of tubercle bacilli. On the one hand, cells present in these cultures vary greatly in age and therefore in physiological state. Heterogeneity of the cell population is further increased when mycobacteria are allowed to grow in the form of pellicles, heaped masses or large clumps; the environmental conditions prevailing in the center of these masses differ greatly from those at the periphery and are probably reflected in structural and metabolic differences between the individual components of the bacterial population. It has been shown that a two week old culture of human tubercle bacilli contains a very large proportion of dead cells[1] and it is likely that many of these have undergone varying degrees of autolysis. This heterogeneity undoubtedly complicates the analysis of the factors affecting the rate of bacterial growth and the establishment of standard experimental infections. Even more probably it obscures the results of immunochemical analysis by leading to the study of artefacts produced during autolysis of the cells, and by preventing the detection and isolation of important cellular components and metabolic products of the normal, physiologically active tubercle bacilli.

In order to obviate the experimental difficulties discussed in the foregoing paragraphs we have attempted to devise a simple culture medium which would allow the growth of inocula containing very few living cells—ideally only one—and to create cultural conditions which would favor homogeneity in the growing culture. The production of large yields of bacterial cells may become another requirement in order to study chemically some components or products of the tubercle bacillus. This requirement, however, need not be satisfied for the solution of the two problems formulated above and may be even incompatible with the production of homogeneous cultures[2,3]. We shall now present in the form of a number of dogmatic statements those of our findings which constitute the theoretical basis of a new medium capable of giving fairly rapid and diffuse growth of minute inocula of virulent tubercle bacilli.

1. *The effect of serum albumin on the growth of tubercle bacilli*

As is well-known, tubercle bacilli can synthesize their structural and metabolic constituents from a few single organic compounds. On the other hand, and

[1] G. S. WILSON and H. SCHWABACHER, Tubercle *17*, 161 (1935/36).
[2] R. J. DUBOS, Proc. Soc. exper. Biol. a. Med. *58*, 361 (1945).
[3] R. J. DUBOS and B. D. DAVIS, J. exper. Med. *83*, 409 (1946).

contrary to general belief, the growth of these organisms is readily inhibited by the presence in the medium of minute concentrations of a variety of substances. This inhibitory effect is usually masked by the practice of using inocula containing large numbers of bacilli, many of which are protected from the environment within compact clumps or pellicles. Inhibition is readily recognized, however, when the inoculum contains only small numbers of cells. We have observed that serum albumin has the remarkable property of antagonizing the antibacterial effect of various toxic substances (fatty acids, heavy metals, chlorine compounds, phenolic compounds, dyes, anionic and cationic detergents, etc.). Albumin permits the initiation of growth of small inocula in synthetic media not by behaving as a nutritional factor but by protecting the bacilli against various toxic effects[1-3].

2. *The effect of wetting agents on the surface properties of tubercle bacilli*

The tendency of tubercle bacilli to grow in the form of compact clumps in ordinary culture media is due, in part at least, to the hydrophobic character of their cell surface. Attempts have been made to increase the wetting properties of this surface by adding to the bacterial suspensions a variety of wetting agents. Unfortunately, all the anionic and cationic surface active substances so far tested have been found toxic to tubercle bacilli. A few non-ionic wetting agents, on the other hand, are of such low toxicity that they can be used for emulsifying the living organisms in aqueous solutions; when added to the culture media they permit submerged or even diffuse growth of all strains of mycobacteria so far tested. One of the non-ionic surface active substances which has proven most useful from these points of view is the emulsifying agent available commercially under the name of Tween 80; some information concerning its nature and properties will be presented in the following paragraphs[1-5].

Tween 80 is an ester of oleic acid—a polyoxyethylene derivative of sorbitan monooleate—which is completely dispersible in water. Whereas the long carbon chain of the oleic acid gives lipophilic properties to this ester, the oxygen-containing groups of the polyhydric alcohol and of the ether oxide chains endow it simultaneously with hydrophilic properties. There is suggestive evidence that Tween 80 becomes adsorbed through its long fatty acid chain on the hydrophobic surface of the tubercle bacillus; the other components of the molecule become so oriented as to form around the organism a hydrophilic layer which permits wetting by the aqueous phase (fig. 1).

In addition to its ability to emulsify the bacterial suspension Tween 80 exerts in many cases a stimulating effect on the growth of mycobacteria. It is likely that wetting of the cell surface facilitates exchanges between the organism and the environment, and thus increases the rate of metabolism. Furthermore, we have obtained quantitative evidence that

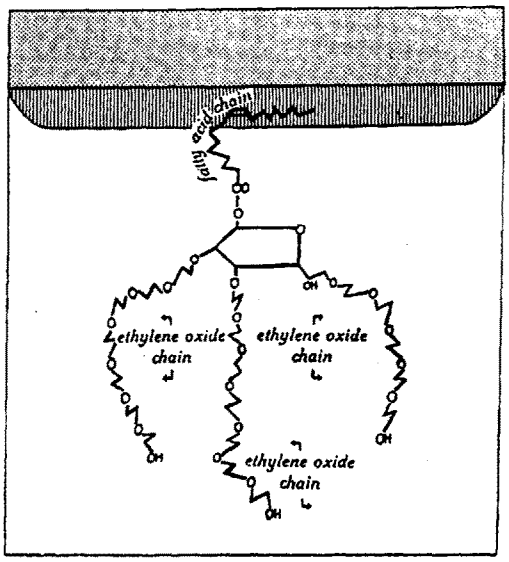

Fig. 1. Adsorption of "Tween 80" on the Tubercle Bacillus through the Long Chain Fatty Acid

Tween 80 can enhance the growth of several bacterial species by contributing to the nutrition of the organisms a readily available non-toxic source of long chain fatty acid[1]. This observation is of sufficient importance to warrant separate discussion.

3. *The effect of long chain fatty acids on the growth of tubercle bacilli*

Tubercle bacilli are extremely susceptible to the toxic action of long chain fatty acids which exert upon them a bacteriostatic and bactericidal effect[1-6]. For example, concentrations of oleic acid as low as 0·000001–0·00001 per cent are sufficient to cause inhibition or retardation of growth of small inocula of human bacilli in synthetic liquid media. On the other hand, esterification of the acids diminishes or abolishes completely their antibacterial action; thus, methyl

[1] B. D. DAVIS and R. J. DUBOS, Arch. Biochem. *11*, 201 (1946).
[2] R. J. DUBOS, Proc. Soc. exper. Biol. a. Med. (1946); J. exper. Med. *85*, 9 (1947).
[3] R. J. DUBOS and B. D. DAVIS, J. exper. Med. *83*, 409 (1946).
[4] R. J. DUBOS, Proc. Soc. exper. Biol. a. Med. *58*, 361 (1945).
[5] R. J. DUBOS, B. D. DAVIS, G. MIDDLEBROOK and C. PIERCE, Amer. Rev. Tub. (1946).

[1] R. J. DUBOS, Proc. Soc. exper. Biol. a. Med. (1946); J. exper. Med. *85*, 9 (1947).
[2] S. BERGSTRÖM, H. THEORELL and H. DAVIDE, Nature *157*, 306 (1946).
[3] C. BOISSEVAIN, Amer. Rev. Tub. *13*, 84 (1926).
[4] B. D. DAVIS and R. J. DUBOS, Arch. Biochem. *11*, 201 (1946).
[5] R. J. DUBOS and B. D. DAVIS, J. exper. Med. *83*, 409 (1946).
[6] W. M. STANLEY, C. H. COLEMAN, C. M. GREEN, J. SACKS, and R. ADAMS, J. Pharm. exper. Ther. *45*, 121 (1932).

oleate, triethanolamine oleate, and a variety of natural and synthetic phosphatides (lecithins and cephalins) do not prevent, and in fact can stimulate the growth of tubercle bacilli. That this decrease in toxicity is not due to poor solubility of the esters appears from the fact that the polyoxyethylene derivatives of oleic acid (purified to remove all traces of unreacted free acid) are essentially non-toxic despite the fact that they are dispersible in water in all proportions and that oleic acid itself is one of the most toxic of long chain fatty acids. Naturally, it must be kept in mind that the esters can be hydrolyzed by lipases present in the medium or produced by the bacterial cells themselves; in other words, it is possible that a non-toxic ester may become inhibitory as a result of saponification during incubation.

We have already mentioned that serum albumin can neutralize the action of many toxic substances. This effect is particularly striking in the case of long chain fatty acids. When an adequate amount of serum albumin is added to an opalescent soap emulsion, there occurs an immediate clearing of the emulsion and a concomitant disappearance of toxicity. It takes approximately 50 parts by weight of albumin to achieve detoxification of 1 part of oleic acid. None of the other proteins tested could replace serum albumin, and moreover the detoxifying power of the latter substance is lost as soon as the integrity of the molecule is destroyed by enzymatic digestion or by heating[1,2].

When rendered atoxic, either by esterification or by admixture with serum albumin, a number of long chain fatty acids (saturated and unsaturated) can enhance the growth of many strains of tubercle bacilli and can in fact serve as a single source of carbon for the growth of these organisms. Tween 80, the water dispersible ester of oleic acid mentioned above, is particularly effective in this respect as appears from the results presented in table I.

4. *Tween-albumin media for the growth of tubercle bacilli*

On the basis of the facts reported in the preceding paragraphs, it has been possible to devise a liquid medium in which submerged growth of small inocula of virulent tubercle bacilli can be obtained within a relatively short time. A satisfactory basal medium can be prepared as follows:

KH_2PO_4	1·0	g	Boil and filter, then add
Na_2HPO_4 12 H_2O	6·25	,,	
Asparagine	1·0	,,	
Enzymatic hydrolysate of casein	2·0	,,	
Ferric ammonium citrate	0·05	,,	
$MgSO_4$	0·005	,,	
Tween 80	0·5	,,	
H_2O	1000·0	cm^3	

The medium can be autoclaved, its final reaction should be p_H 6·5–7·0.

Following inoculation of 5 ml of this medium with 0·01–0·001 mg of tubercle bacilli, macroscopic evidence of growth can be obtained after 2 days incubation at 37°C in the case of the avian strains, and after 3–4 days in the case of the human strains. An abundant and diffuse growth consisting of individual cells and of microscopic clumps is usually obtained after 5–7 days incubation. For the reason mentioned earlier, development of very small inocula usually requires the addition to the medium of 0·1–0·5% serum albumin (introduced aseptically in the form of a 5% solution sterilized by filtration through glass or porcelain filters). In the presence of this protein, evidence of growth can be obtained within 10–14 days following inoculation of 5 cm^3 of medium with 10^{-8} mg bacilli.

Table I

Effect of Albumin and of Oleic Acid and its Esters on Bacterial Growth in Casein Hydrolysate Liquid Media

Lipid added to medium		Growth (mg/10 ml) in media containing			
		No. albumin		0·5 per cent albumin	
		Avian TB	Human TB	Avian TB	Human TB
	per cent	mg	mg	mg	mg
Oleic acid	0·01	0	0	1·6	2·1
	0·003	0	0	0·5	1·4
	0·001	0·3	0	0·3	0·9
	0·0003	0	0	0·3	0·8
	0·0001	0	0	0·3	0·9
Methyl oleate	0·1	1·5	0	0·9	2·4
Polyoxyethylene derivative of sorbitan monooleate Tween 80	0·1	2·5	0	3·8	3·0
	0·03	0·9	1·4	1·3	1·7
	0·01	0·3	0·7	0·3	0·8
	0·003	0·1	0·9	0·3	0·9
	0·001	0	0·1	0·3	0·6
Control	—	0	0·1	0	0·4

By melting 2% agar with the basal medium and adding enough serum albumin to give a final concentration of 0·5% it is possible to prepare a solid medium which is satisfactory for colonial development of tubercle bacilli. When cultures growing diffusely in the liquid medium are inoculated on the surface of Tween-albumin agar, the number of colonies which become visible within 10–12 days correspond to a bacterial population of approximately 10^8 living cells per cm^3 of liquid culture. It appears likely, therefore, that almost every cell, or clump of cells, gives rise to a colony, and that, under certain conditions, the agar

[1] B. D. DAVIS and R. J. DUBOS, Arch. Biochem. *11*, 201 (1946).
[2] R. J. DUBOS, Proc. Soc. exper. Biol. a. Med. (1946); J. exper. Med. *85*, 9 (1947).

method can be utilized for quantitative cell counts[1]. Mention may be made at this time that marked differences in colonial morphology have been observed between different cultures and within one given culture; however, no information is as yet available to correlate this evidence of bacterial variability with other alterations of biological or immunochemical properties.

structure of the tubercle bacillus and in providing additional tests to follow the course of the disease.

The cultures possess a high degree of virulence for guinea pigs and moderate virulence for the mouse and the developing chick embryo. Infection of the latter via the yolk sac with 0·01 mg bacilli causes the appearance on the chorio-allantoic membrane of large numbers of gross tuberculous lesions with a characteristic hematogenous distribution (fig. 2–3).

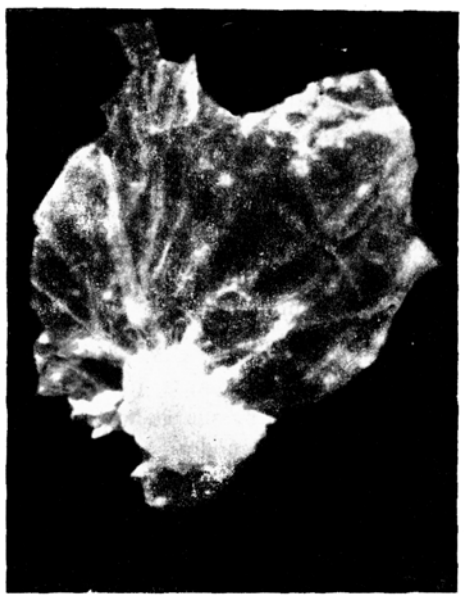

Fig. 2.

Fig. 3.

Fig. 2. Chorio-allantoic membrane of the developing chick embryo days after direct inoculation of the surface of the membrane.
Membrane inoculated on the 10th day of incubation with 0·05 mg human tubercle bacilli. (Strain: H37S cultured in liquid medium containing 0·1 % Tween 80, 0·1 % serum albumin.)

Fig. 3. Chorio-allantoic membrane of the developing chick embryo 10 days after yolk sac inoculation.
Yolk sac inoculated on the 7th day of incubation with 0·015 mg human tubercle bacilli. (Strain: DUBOS 1 cultured in liquid medium containing 0·1 % Tween 80, 0·1 % serum albumin.)

Properties of tubercle bacilli growing diffusely in liquid media

Avian, bovine and human tubercle bacilli growing in the presence of water soluble esters of oleic acid exhibit the usual morphology and staining characteristics of mycobacteria. They retain their viability for long periods of time, in particular when serum albumin is added to the medium; thus cultures of the human strain H37Rv maintained for 2 months at 37°C in liquid medium (0·05% Tween 80 and 0·2% albumin) contain 10^8–10^9 living bacilli per cm^3 as determined by colonial counts on agar, or by the dilution method in the same liquid medium.

These submerged cultures permit the preparation of homogeneous and stable bacillary suspensions which can be used for agglutination tests with human and animal sera (table II)[2]. It is possible that these reactions will be helpful in the analysis of the antigenic

Table II

Serological specificities in agglutination reactions of avian (KIRCHBERG) and mammalian (H37Rv) strain tubercle bacilli grown in "Tween 80" medium

Serum dilutions	Normal pre-immune rabbit serum		Anti-avian rabbit serum		Anti-avian rabbit serum absorbed with H37Rv	
	KIRCHBERG	H37Rv	KIRCHBERG	H37Rv	KIRCHBERG	H37Rv
1:20	–	–	+++	+	++	–
1:40	–	–	+++	+	++	–
1:80			+++	+	+	–
1:160			+++	+	+	–
1:320			++	–	+	–
1:640			+		–	–
1:1280			–		–	–
1:2560			–		–	–
1:5120			–		–	–
saline control	–	–				

(Agglutination mixtures incubated at 50° C for 3 hours, then overnight at 38° C.)

[1] The addition of glycerol to the Tween medium often inhibits the multiplication of small inocula and never increases the initial rate of growth; small amounts of glucose increase the yields of bacilli, but do not affect the initial rate of multiplication.

The growth of all avian strains so far tested is greatly enhanced by the addition of Tween 80 to the medium. A few human and bovine strains have been found to be slightly inhibited by the substance.

[2] R. J. DUBOS, B. D. DAVIS, G. MIDDLEBROOK and C. PIERCE, Amer. Rev. Tub. (1946).

Table III

The Effect of the Egg Yolk Factor on the Infection of White Mice with Human Tubercle Bacilli growing diffusely in Tween-albumin Medium

Amt. injected i.p.		Results after 3 weeks infection						
		Death survival ratio	Average weight of mice				Gross pathological findings	
Culture H37S	Egg Yolk		Initial	1 week	2 weeks	3 weeks	Average weight of spleen	Macroscopic appearance of tissues
mg	cm^3		gm	gm	gm	gm	mg	
0	0	0/6	18·8	19·4	20·8	21·8	97	normal
0	0·12	0/6	20·0	19·9	20·5	22·5	75	normal
0·03	0	0/6	20·0	20·2	22·6	24·2	740	enlarged lymph nodes pinpoint pulmonary lesions occasional scattered abscess-like lesions throughout other tissues
0·03	0·06	1/6	20·2	20·1	20·3	20·3	358	enlarged lymph nodes extensive pulmonary lesions few other lesions
0·03	0·12	6/6	20·5	20·7	18·0	all dead	—	—
0·15	0	0/6	21·0	20·3	21·7	22·6	553	enlarged lymph nodes pinpoint pulmonary lesions numerous scattered abscess-like lesions throughout other tissues
0·15	0·12	6/6	21·4	all dead	all dead	all dead	—	—

Since the mouse has become one of the most convenient and widely used laboratory animals for the study of experimental infections, it may be worthwhile to discuss in greater detail the response of this rodent to the injection of tubercle bacilli grown in the Tween-albumin liquid medium.

Factors affecting the susceptibility of mice to tuberculous infections

It has long been known that one can establish a fatal infection by injecting adequate numbers of tubercle bacilli into mice. Published evidence indicates that inhalation of fine emulsions (aerosols) of the organisms constitutes the most efficient method of inoculation[1]. To be effective, injection by the i. v. or i. p. route requires huge infective doses when the organisms are obtained from the classical culture media, and moreover, the results of the infection are even then often irregular. We have observed, however, that white mice (3—6 weeks old) die within 4 weeks with extensive pulmonary lesions following injection into the caudal vein of 0·1 cm^3 of a 7 days old culture of virulent human strains growing diffusely in the Tween-albumin medium. This infective dose contains approximately 0·015 mg dry bacilli and corresponds to considerable numbers of living organisms. We have attempted, therefore, to determine whether the minimal infective dose could be decreased by the addition of certain substances to the bacteria.

Although i. p. injection of tubercle bacilli is much less effective and gives less reproducible results than infection by inhalation or by the i. v. route, we have selected the first method for the preliminary phase of our studies because of its convenience and rapidity of performance. It has thus been found that the ability of tubercle bacilli to establish an infection in mice is much enhanced when the organisms are mixed with certain types of organic materials before injection into the animal. Egg yolk has been, so far, the most effective of all the adjuvants tested, and its effect on the course of infection can be recognized by a number of different criteria: weight curve and survival time of the infected animal, degree of enlargement of the spleen after different periods of infection, number and size of the pulmonary lesions, etc. All these criteria are affected simultaneously by the addition of the egg yolk factor to the infective dose except for the fact that enlargement of the spleen may not become manifest when the disease runs a fairly acute course (death within 2—4 weeks) or when pulmonary lesions are extremely extensive (table III).

The enhancing effect of egg yolk on infection is not limited to the i. p. route, but becomes even more striking when the mixture egg yolk and bacteria is

[1] R. D. GLOVER, Brit. J. exper. Path. *25*, 141 (1944).

injected into the caudal vein of the mouse. As will be shown in forthcoming publications, mice inoculated under these conditions with 0·01 mg bacilli die within 2 weeks with extensive pulmonary disease.

It is also worth mentioning that cultures of mycobacteria known to be devoid of pathogenicity for guinea pigs (saprophytic mycobacteria, avirulent variants of human and bovine strains) fail to cause disease in mice, even when very large amounts of organisms are injected in mixture with amounts of egg yolk known to be optimal for the enhancement of pathogenicity of the virulent forms.

Although age did not appear to modify appreciably the response of our animals to injection of tubercle bacilli, marked differences were observed between different breeds of mice with reference to the distribution and severity of the tuberculous lesions. The state of nutrition also appears to affect the course of the disease since, in two different experiments, animals kept on a poor diet (comprising a very large proportion of starch and gelatin) developed more numerous and extensive pulmonary lesions than animals maintained on a more complete diet.

Discussion

One may question the wisdom of attempting to study the natural history of tuberculosis--a chronic disease—by changing the rate and mode of growth of its causative agent, and by producing an experimental infection with a rapid and almost acute course. It should be pointed out in this respect that, although the chronicity of the reparative processes of tuberculosis is usually emphasized, the progressive phases may be, and frequently are, acute. This is true not only in the early stages, before immunity and hypersensitiveness have become established, but also in advanced pulmonary tuberculosis in the adult. "—exudative processes may under certain conditions have an acuity similar to those of pneumococcic pneumonia. The development: invasion of fresh tissue—pneumonic infiltration—excavation may take place within a week"[1]. Studies of accelerated growth rates of tubercle bacilli and of the acute processes which can be produced in experimental animals are not, therefore, irrelevant to an understanding of the natural disease.

It need not be stated that Tween 80 is a completely synthetic product which does not exist in nature and that its addition to culture media is a laboratory artifice. On the other hand, there are present in animal tissues a variety of water dispersible esters of long chain fatty acids—phosphatides for example—which have many of the physicochemical properties of Tween and which, like this synthetic substance, may wet the hydrophobic surface of the tubercle bacillus, accelerate the rate of its metabolic exchanges, and provide it with non-toxic readily available long chain fatty acids. It is worth recalling in this respect that tubercle bacilli inoculated into diluted, unheated egg yolk give rise to submerged and diffuse growth[1], a fact which suggests that there exist in tissues substances which can wet the hydrophobic bacterial surface.

It is possible, therefore, that the Tween-albumin medium does not constitute as unnatural an environment as would appear from its composition. In any case, this medium was devised to provide conditions favorable for greater homogeneity of growth and for better survival of the cells. It is hoped that cultures grown under these conditions will provide more satisfactory material for the separation in an active form of those cellular components and products which condition the response of the host to tuberculous infection.

The disease induced in mice by the i. v. or i. p. injection of tubercle bacilli growing diffusely in Tween-albumin medium corresponds to an overwhelming hematogenous invasion in an animal possessing a fair degree of natural resistance, but no acquired immunity or hypersensitiveness. Naturally this experimental disease differs profoundly from human tuberculosis both in its course and its pathology. Granted these differences, it remains possible and likely that the mouse disease can be a useful tool for the analysis of certain phases of tuberculous infections. It is sufficient to recall that the immunology and epidemiology of yellow fever, as well as its control by a practical vaccination technique, have been worked out with the experimental encephalitis caused by i. v. injection of the virus into white mice, a disease, the course and pathology of which bears no relation to that which occurs in man. Similarly in the case of pneumococcus lobar pneumonia, the understanding of immunity processes, and the discovery of the most effective chemotherapeutic agents, have resulted from the study of experimental pneumococcus peritonitis in the mouse, again a disease very different from pneumonia in man. By analogy one may hope, therefore, that the acute infection of mice with tubercle bacilli will lend itself to the investigation of certain aspects of the immunology and chemotherapy of tuberculosis. It is obvious, that different types of experimental infection, and different species of experimental animals, may be more favorable for the study of other aspects of the disease—in particular, of those which are dependent upon the hypersensitive state. Nevertheless, the phenomena reported in the present paper appear sufficiently striking to warrant

[1] M. PINNER, Pulmonary Tuberculosis in the Adult. Charles C. Thomas, 1945.

[1] A. BESREDKA, Culture des bacilles tuberculeux dans du jaune d'œuf, Ann. Inst. Pasteur *35*, 291 (1921).

the conclusion that the Tween-albumin medium, and the acute tuberculous infection in the mouse, will prove useful for the discovery and analysis of some of the factors which affect the course of tuberculosis in man.

Zusammenfassung

Züchtet man Tuberkelbazillen nach den bis heute üblichen Methoden, so begegnet man immer wieder gewissen Schwierigkeiten, die sich nicht leicht beheben lassen: Die Bakterien wachsen nur sehr langsam; sie bilden Klumpen oder kompakte, an der Oberfläche der Kultur schwimmende Häute, die aus einem uneinheitlichen Gemisch verschieden alter, lebender und toter Bakterien bestehen; sie lassen sich nicht gut homogen in einer Aufschwemmung verteilen. Außerdem kann man nur sehr große Inocula mit Erfolg verimpfen, da kleinere Bakterienmengen in der Regel nicht angehen.

Diese Umstände erschweren das experimentelle Arbeiten in vielerlei Hinsicht, und es wurde deshalb ein Züchtungsverfahren ausgearbeitet, welches es gestattet, ausgehend von sehr kleinen Inocula (10^{-8} mg Bakterien) Kulturen zu bekommen, die sich in flüssigen Nährmedien innert weniger Tage unter homogener Trübung des Milieus entwickeln. Der wesentliche Bestandteil dieses neuen Mediums ist ein nichttoxisches Netzmittel, ein Ölsäureester, und zwar ein Polyoxyäthylenderivat von Sorbitmonooleat (Markenname «Tween 80»). Dieser Stoff haftet mit Hilfe einer hydrophoben Gruppe am Tuberkelbazillus, mit seinen langen Alkoholketten macht er aber den Bakterienleib nach außen hydrophyl, so daß er in der wässerigen Lösung frei und homogen suspendiert bleibt und zudem leichter Nährstoffe aus der Lösung aufzunehmen vermag. Außerdem scheint die veresterte Ölsäure selbst auch das Wachstum zu fördern.

Die so gewachsenen Tuberkelbazillen behalten ihre Virulenz über lange Zeit bei. Ferner lassen sie sich durch Immunseren agglutinieren, was neue diagnostische Möglichkeiten eröffnet. Außerdem ist es möglich, damit Hühnerembryonen und besonders Mäuse zu infizieren, die einen andern Typ von Tuberkulose entwickeln als nach Infektion mit gewöhnlich gewachsenen Tuberkelbazillen, nämlich eine rasch tödlich verlaufende Lungentuberkulose. Obwohl diese akut verlaufende tuberkulöse Infektion von zahlreichen Formen der menschlichen Erkrankung wesentlich verschieden ist, wird doch die Hoffnung ausgesprochen, daß damit dem Experimentator eine neue, praktische Versuchsanordnung zum Studium der Tuberkulose in die Hand gegeben sei.

Einige Bemerkungen zum Wasserhaushalt der Wassertiere

Von W. v. BUDDENBROCK, Mainz

Obgleich der Wasserhaushalt ein integrierender Bestandteil des Stoffwechsels ist, hat er einen völlig anderen Charakter als die übrigen hierher gehörigen Prozesse. Bei der Verarbeitung der organischen Nährstoffe ist das Wesentliche der Wechsel. Da der Körper fortwährend Energie verbraucht, müssen ihm fortwährend neue Nahrungsmengen zugeführt werden, die die entsprechenden Kalorien enthalten. Stets erneute Nahrungsaufnahme, Verarbeitung derselben und Ausscheidung der Reste sind daher lebensnotwendige Prozesse für alle Organismen, die irgendwelche Arbeit verrichten.

Beim Wasserhaushalt ist dagegen die Aufrechterhaltung des normalen Wassergehalts des Körpers die Hauptsache. Ein fortwährender Wechsel des Wassers, wie wir ihn bei den Nahrungsstoffen sehen, ist daher gar nicht erforderlich. Es gibt Organismen, bei denen ein solcher Wechsel fast gar nicht existiert. Für gewöhnlich ist es aber so, daß beständig bestimmte Kräfte bestrebt sind, den normalen Wassergehalt des Körpers zu ändern, sei es, daß sie ihm Wasser entführen, das ersetzt werden muß, sei es, daß Wasser in den Körper dringt, dessen Entfernung notwendig ist. Das erste geschieht bei den Landtieren, das zweite bei den Wassertieren.

Ein großer Teil dieser Faktoren hat gar nichts mit dem eigentlichen Stoffwechsel zu tun. Die Wasserverluste unseres Körpers gliedern sich zum Beispiel in Verdunstung, Schwitzen, Ausatmung feuchter Luft und Harnabgabe. Nur dieser letzte gehört zum engern Kreis der Stoffwechselvorgänge. Die Verdunstung ist eine einfache Folge der physikalischen Beschaffenheit unserer Haut, der Schweißverlust dient der Abkühlung unseres Körpers, die Ausatmung von Wasser durch die Lunge ist eine Folge des Atmungsprozesses. Bei den Wassertieren wird das fortwährende Einströmen des Wassers durch die Haut durch osmotische Kräfte bewirkt. Auch dieser Vorgang steht den eigentlichen Stoffwechselvorgängen gänzlich fern; er ist eine Folge des verschiedenen Salzgehalts des Außenmediums und des Innenmediums sowie der Durchlässigkeit der Haut.

Den ursprünglichsten und einfachsten Fall stellen ohne Zweifel die wirbellosen Tiere des Meeres dar: Würmer, Schnecken, Muscheln, Krebse usw. Von einem richtigen Wasserhaushalt ist bei ihnen kaum zu reden. Sie nehmen etwas Wasser durch die Nahrung auf, und eine entsprechende Menge wird durch den Harn abgegeben. Dies ist alles. Es wird kein Wasser getrunken und es gibt keine größeren Wasserverluste. Das Blut dieser Tiere ist in seiner Zusammensetzung dem Meerwasser außerordentlich ähnlich; es enthält die Salze in der gleichen Menge oder ist, wie man zu sagen pflegt, zum Seewasser isotonisch. Es sind daher gar