THE WIDER ASPECTS OF THE CHEMOTHERAPY OF TUBERCULOSIS

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I. INTRODUCTION

The recent history of bacterial chemotherapy has so influenced our philosophy and our science, that it is usual to discuss the subject in terms of simple relations between bacteria and chemical; often it has seemed unnecessary to acknowledge the host. Indeed, applied to tuberculosis, this simple approach has been proved by the test of experience. No doubt, the application of these empiric methods, which we shall discuss, has yet to yield greater triumphs. But, their limitations are now apparent. No other host-parasite relation has been the subject of such detailed enquiry at the hands of pathologists (130), bacteriologists, chemists, (165, 1) and others. These studies have emphasised the most destructive nature of the parasitism, the remarkable collaboration of parasite and host in the death of the host's vital tissues, and the unsolved riddle of delayed lysis of caseous tissue with its capacity to spread infection. Appreciation of the unusual pathology of this normally self-limiting chronic disease has emphasised the desirability of approaching the problem of elimination of the parasite on a broad front. It will be convenient for our present purpose first to describe the nature of the biochemical lesions which the observed pathological changes indicate, and from this basis to review the present advances.

Usually, by the time the disease is identified clinically it has established its characteristic pathology, the tubercle, and this is so even in its more acute manifestations (131). The minimal clinical lesion visualised, for example, by X-ray shadows, represents destructive changes and reflects, as we shall see, at least two biochemical lesions.

II. THE ORIGIN OF THE TUBERCLE AND ITS ULTIMATE FATE

The tubercle is the characteristic but not unique lesion of the disease: typical epithelioid cell tubercles are seen in sarcoid, leprosy, tularemia, schistosomiasis, and syphilis. Many studies of all stages of the development of tubercles enable an unequivocal description to be given (130). Within a few minutes of an experimentally induced infection, a normal mononuclear phagocyte ingests a (single) invading tubercle bacillus. The cell increases in size, its nucleus enlarges, and it becomes an epithelioid cell (135). Next, fusion of several epithelioid phagocytes takes place to give a Langhans giant cell with its numerous nuclei arranged in a "rosette", or there may be formed the less frequent "foreign-body giant cell" in which the nuclei are scattered. Within 3 or 4 days a small nodule is built up by clustering epithelioid cells, shown by Metchnikoff (154) to be simply altered monocytes. Since the nodule grows progressively after access to blood-borne phagocytes is cut off, it is believed (130) that the phagocytes multiply *in situ*. Gradually the adjacent tissue cells are pushed aside, dying from nutritional deficiencies caused by pressure of the expanding tubercle. At this stage of development collagen fibres are formed between the epithelioid cells. Should multiplication of the bacillus be held in check, no necrosis is observed, and with the death of the tubercle bacilli the transfer to collagen becomes progressive, the tubercle becoming fibrous so that only a nodule of fibrous tissue finally

remains. Complete resolution and disappearance of the tubercles have been frequently reported, and this is also true of large tubercles which had proceeded to central caseation in guinea-pigs (60). Should multiplication proceed, the central portion dies and becomes necrotic. Recently, the tissue reactions seen when tubercle bacilli are introduced through a removable plug in the back of a transparent chamber in a rabbit's ear have been described (46). These direct observations made under the microscope confirm and extend the older histological studies.

Permeability of the Tubercle. Important for any consideration of chemotherapy is whether tubercles and associated caseous tissues are readily permeable. The chemical should also pass into the tubercle bacillus, and into phagocytes if the bacilli are intracellular. Rich (130) points to the inherent evidence that viable tubercle bacilli and host cells depend on diffusible nutrilites for their continued survival and to the frequency with which central foci become calcified. Also there is now a wealth of presumptive evidence in both the experimental animal and man that sulphones, streptomycin, para-aminosalicylic acid (PAS) thiosemicarbazones, and isoniazid diffuse into tubercles. Brownlee found sulphetrone to penetrate normal and caseous tissues of man with equal facility (24) but the dynamics of the diffusion were quite unknown. Precise information has been lacking until quite recently about penetration of substances into phagocytes. Mackaness (97) has devised an elegant technique whereby rabbit macrophages are offered bovine tubercle bacilli (Branch strain) which have been grown for three weeks. Engulfed by the rabbit macrophage, the coccobacillary forms can be seen first to grow in length, and then to multiply. After forty-eight hours the macrophage dies, liberating the bacilli into the surrounding medium. Drugs can be applied to the macrophages and the effects on the bacilli can then be observed.

When proved antituberculous drugs are examined in this way several important facts emerge. The first is that much higher concentrations are needed to inhibit the growth of intracellular bacilli than are required to protect the rabbit from the experimental disease. The next point is that one proved antituberculous drug, PAS, does not inhibit intracellular bacilli even at enormous concentrations; that another, streptomycin, inhibits at higher concentrations than are needed for extracellular organisms; and that terramycin is as effective against intracellular as extracellular organisms. Only isoniazid is able to bring about the death of intracellular bacilli (98).

It would seem logical to extend Mackaness's conception of protection of bacilli within macrophages to protection within their progeny, the epithelioid cells. When bacilli are uninfluenced by chemotherapy, it may be because the drug does not reach them, or because the bacilli are already metabolically inactive and so are in an insensitive state. The concept of a resting phase was suggested by Koch (84) and the same idea was canvassed by Jensen (78).

Hypersensitivity and Necrosis. Although the death of host cells appears to follow to some degree the lodgment of the foreign body, which the tubercle also is, it is now known that the widespread tissue destruction which characterises the dis-

ease follows the conditioned hypersensitivity induced by the products of metabolism of virulent pathogens. The protein component is known to be responsible and appears to be identical with tuberculin which is harmless to the normal insensitive animal but is a deadly poison to the sensitised host. All that is known of this mechanism confirms the opinion that the clinical disease in all its manifestations follows from the phenomenon of hypersensitivity. Actively multiplying organisms appear to be essential, and virulence in this connection connotes a capacity to multiply. The role of the lipopolysaccharide in acting together with tuberculin to sensitise the host is discussed elsewhere.

Caseation. Liquefaction of body cells after their death is the normal prerequisite for disposal by phagocytes and is a function of proteolytic enzymes contained in the cells. In the characteristic tuberculous lesions, only partial autolysis occurs, and the necrotic cells lose structure, outline and nuclei to become, together with their intercellular materials, a formless "caseous" mass. Opinion is divided on the reason for incomplete digestion. Opie and Barker (cited in 21) showed that active enzymic function could be identified with the onset of caseation but subsequently ceased, whether because of absence or inactivation was not proved. Jobling and Petersen (79) found that the soaps of unsaturated fatty acids extracted from tubercle bacilli inhibited *in vitro* the proteolytic activity of the leucocytic enzymes. On the other hand, caseation is characteristically observed in man in infections with micro-organisms which attract mononuclear phagocytes, for example, in typhoid but not in the allied colon bacillus infections which attract polymorphonuclear phagocytes. It is of great interest that typical caseation follows the necrosis of "lipoid pneumonia" which, for example, results from the accidental introduction of cod liver oil into the human lung. An outpouring of mononuclear phagocytes also characterises these lesions (130). The predominance of mononuclear phagocytes in caseous lesions induced the comparison of Weiss and Czarnetzky (170) between the proteolytic enzyme activity of the two types of cell. The monocytes of rabbits contained one proteinase, pepsin, with an optimal activity at pH 3, whereas the polymorphonuclears contained pepsin, cathepsin and trypsin, with optima at pH 3, 5.4 and 8, respectively.

"Softening." The "softening" of caseous lung substances allows imprisoned tubercle bacilli to be discharged into the air-passages and thus to infect new sites. In contrast to caseous areas, a remarkable characteristic of softened areas is the large number of tubercle bacilli they contain. It seems logical to attribute the lysis of caseous material to enzyme action but this is unproven. The current view appears to attribute the renewed digestion to the activity of infiltrating polymorphonuclear leucocytes which are commonly identified in freshly softening areas (75, 130), since acid-fast tubercle bacilli are known to be poor in proteolytic enzyme content (165). An alternative explanation (19) is that the micro-organisms which cause the extensive lesions of the early stages of infection are unusual in form and appearance and do not stain acid-fast. Indeed it is true that conventional staining methods indicate that many more macrophages seem to be infected than contain acid-fast bacilli. Applying new tools to an old task, Brieger and Glavert (19) have identified several atypical phases of growth of an avian strain of a tubercle bacillus with the aid of phase contrast and electron

microscopy. Two prebacillary forms seem of interest; the first were branched mycelia which do not stain acid-fast; the second were forms, spherical and with finger-like protrusions, similar to the large viruses when viewed by the electron microscope. Tissue extracts containing these forms could be obtained seemingly free from any bacillary elements; indeed acid-fast growth could not be obtained on artificial media, yet these fluids infected guinea pigs with classical tuberculosis. Obviously, the alternative explanation, that a few bacilli escaped observation and did not grow on artificial medium because their growth requirements were not met, will have to be rigorously excluded.

Fate of the Caseous Lesion. Should the tubercle bacilli die, the caseous area may become encapsulated by connective tissues, or it may be completely resolved. This surprising observation is now well documented (167, 117).

Calcification. The calcium phosphate which is deposited in caseous areas has the same composition, $Ca_3(PO_4)_2$, as that of normal bone (165), and apart from the suggestive indications that high serum calcium and phosphorus concentrations influence calcium deposition (26) as, for example, in children generally, and that phosphatase (26) and vitamins A (105) and D play an important but as yet undisclosed part, no final comment can be made on the conditions governing deposition of calcium in necrotic tissue.

III. THE CHEMICAL COMPOSITION OF TUBERCLE BACILLI AND THE BIOLOGICAL ACTIVITY OF THEIR COMPONENTS

The status of our knowledge concerning chemical composition up to 1932 is collected and admirably summarised by Wells and Long (165). Subsequent to this, Sabin (134) reported the biological effects produced by the lipins fractionated by Anderson (1) and his colleagues from standard strains of acid-fast bacteria grown under carefully standardised conditions of synthetic media, choice of containers and conditions of growth. A complete bibliography of this work is available (171). Anderson (1) fractionated under CO_2 the lipin fractions from acid-fast bacteria into a phosphatide extracted by alcohol-ether, an acetone-soluble "fat", and a chloroform-soluble "wax". The "wax" fraction is a complex phosphatide; it separates into a high and a low-melting fraction. The high-melting fraction is hydrolysed with difficulty to give an acid analogous to phthioic acid and a polysaccharide; there is also phosphorus and glycerol. The other fraction yields numerous glycerides of saturated fatty acids of the phthioic acid series. The acetone-soluble "fat" contains neither phosphorus nor nitrogen, and yields a carbohydrate and numerous fatty acids on hydrolysis. The acids present are butyric, palmitic, stearic, cerotic, linoleic, linolenic, tuberculostearic and phthioic. This acetone fraction proved to be the best source for the characteristic fatty acids of the tubercle bacillus which are present in predominating amounts.

From the acid hydrolysis of the phosphatide has been obtained a homologue of stearic acid, named tuberculostearic acid, subsequently shown by Spielman to be 10-methylstearic acid, and found to be without biological activity (134). There were also separated (+)- and (−)- hexacosanic acids, $C_{26}H_{52}O_2$, named phthioic acid, of which only the (+)- acid had biological activity (134).

Analogous but optically inactive acids with biological activity (134) were

extracted from the lipins of avian and bovine tubercle bacilli, and from leprosy bacilli and timothy-grass bacilli (1). There was also evidence of small amounts of higher acids. More recent evidence indicates the presence in human tubercle bacilli of many branched-chain fatty acids not hitherto identified. Polgar (122) has presented an improved scheme for separating the acids, which are first converted into acetonyl esters and then into semicarbazones which are then crystallised; from this treatment four new acids emerge. Recent evidence pointed to the likelihood that Anderson's phthioic acid was a mixture, probably of two acids.

Acid-fastness. A large group of organisms, of which the tubercle bacillus is one, resist decolorisation with acids after being dyed with aniline dyes. This property is retained by the "waxy" fraction, and of that complex by an acid of high molecular weight named "mycolic acid" (1). Certain evidence points to the fact that the physicochemical state of mycolic acid within the bacillus contributes to acid-fastness (12, 150).

Biological Properties of the Isolated Lipins. In the hands of Sabin and her colleagues (136, 138, 134) all three of Anderson's (1) lipin fractions, but no other fraction, protein or carbohydrate, produced tubercles. Of these the phosphatide was most active in giving epithelioid giant cells and subsequent caseation, and this applied to phosphatide from human, avian, bovine, timothy-grass and lepra bacilli, in that order of biological activity (134). The only other substance among the controls which "acts just like the tuberculo-phosphatide" (134) is lecithin. Tuberculostearic acid was found to be irritating but did not produce tubercles. (+)- Phthioic acid but not (−)- phthioic acid produces typical tubercles. Sabin (134) has refuted the suggestion of Boissevain and Ryder (18) that bacillary debris accounted for the phosphatide activity. Still others (17) are critical of the specific activity of the phosphatide being attributable to phthioic acid. Of more moment is the criticism of Rich (130) who points to the disproportionate amounts of phosphatide and phthioic acid needed to produce tubercles and caseation compared with the observable depredations of a single bacillus. For example, the phosphatide from as much as 300 mg. of bacilli produced only a little caseation in 1 of 4 guinea-pigs injected intraperitoneally, and the amount had to be increased to that extracted from as much as 8.0 gm. of bacilli before each of two guinea-pigs injected developed caseous reactions (152). More recently Ungar, Coulthard and Dickenson (164) found the synthetic 3:13:19-trimethyltricosanoic acid of Polgar and Robinson to be more active than crude (+)- phthioic acid from human tubercle bacilli in the production of tubercle-like granulomata which "corresponded in some respects to the description by Sabin, Doan and Forkner" (136). Of 15 synthetic acids tested, 10 were as active as or more active than the natural product. The most active synthetic substance was 3:12:15-trimethyl-docosanoic acid which showed granulomata with as little as 10 to 25 mg. in a single intraperitoneal dose suspended in aqueous alcohol. The surface layer of precipitated phthioic acid analogues is no doubt very different from that of the continuous film of the intact bacillus. Realisation of this fact prompted Ungar (21) to observe the chemiotactic response of macrophages to agar blocks in which these acids were entrained and then subsequently

implanted in the peritoneal cavity of guinea-pigs. The first cells to penetrate were polymorphonuclear leucocytes and lymphocytes; then came monocytes which engulfed the particles and became typical epithelioid cells. Ungar has made some preliminary observations which if confirmed will shed light on the quantitative aspect of the responses of phthioic acid and its analogues. He coated killed colon bacilli with 3:13:19-trimethyltricosanoic acid, and injected the suspension intraperitoneally into guinea-pigs; the subsequent granulomata of the omentum and elsewhere were indistinguishable from those in response to killed tubercle bacilli simultaneously injected into controls, and differed entirely from the minor reactions observed in control animals injected with suspensions of colon bacilli.

The lipopolysaccharides isolated by Lederer (88) have produced similar lesions; the importance of this observation lies in the fact that these contain the mycolic acids as they are present in the bacilli.

As far as the evidence goes we may claim that the cell reactions which result in tubercle formation are non-specific, and probably due to branched-chain fatty acids.

Biological Significance of the Polysaccharide Complexes. The tuberculocarbohydrates isolated by Johnson, Coghill, Brown and Renfrew (134), the polysaccharides isolated from the lipins by Anderson (1) and the carbohydrate isolated from media by Long and Seibert (134) were studied by Sabin (134). Their biological activities appeared to be restricted to a chemiotactic and damaging effect on leucocytes. They have no power to induce hypersensitivity (137) but can act as haptens (86). That is to say, unlike the pneumococcus-specific carbohydrate (4, 53), the tuberculocarbohydrate does not stimulate antigen formation or induce protection, but is capable of reacting in precipitin tests with sera from infected hosts (86). A critical biological re-examination of the three different specific polysaccharides is overdue. Heidelberger and Mizel (21) found the principal serologically active cell component ($[\alpha]_D^{18°}$ + 85°) to contain *d*-arabinose and *d*-mannose, and later found a second specific somatic polysaccharide of lower dextrorotation (21). Haworth, Kent, and Stacey similarly isolated a somatic polysaccharide of $[\alpha]_D^{18°}$ + 85° together with a deoxyribonucleic acid derivative and glycogen and, also, a polysaccharide of $[\alpha]_D^{18°}$ + 25° closely associated with the cell lipins. A recent detailed review by Stacey and Kent should be consulted (155) and there is a competent immunological review by Burger (27).

The extraction procedures of Anderson and his colleagues are convenient and have been followed by others. They dictate the treatment of the subject under the headings: carbohydrates, lipins and proteins. Yet the most active compounds are complexes containing all three. These are now being studied. Stacey and his collaborators (156) have described the properties of lipopolysaccharides extracted from heat-killed bacilli with solutions of urea and β-hydroxypropionamidine. Both preparations are antigenic. Choucroun (34) has isolated a similar lipopolysaccharide called PMKo from heat-killed and dried bacilli with "Vaseline oil", and Asselineau and Lederer (3) have obtained a similar fraction from "purified wax". These are the materials which, injected together with old tuberculin or its purified products, sensitise the animal to tuberculin. Human virulent

strains contain a greater amount of this complex than non-virulent bacilli; in addition there is some evidence that non-virulent variants and BCG have lower melting lipopolysaccharides, with no amino-acids but ammonia instead (88).

Biological Significance of the Proteins of the Tubercle Bacillus. A number of proteins have been extracted from the tubercle bacillus (1) and one from the medium in which it is grown (165). In their purest forms these proteins have practically no toxicity for the uninfected body (147) but are lethal in extremely small doses for the tuberculous subject, as was first demonstrated by Koch for impure "tuberculin" (85). The innocuous nature of purified tuberculoprotein for non-sensitised cells was demonstrated in an elegant way in tissue-culture preparations by Rich and Lewis (21) and contrasted with its lethal effect on similar cells from tuberculous hypersensitive animals (2, 111). Thus, although innocuous for the normal, it is highly toxic for the tuberculous hypersensitive body, causing necrosis, fever, severe constitutional symptoms and even death (130). These facts, first demonstrated by Koch (85), have been repeated many times (95) and have been redemonstrated in a most convincing fashion by Seibert (95) using "pure" tuberculin (145). The protein itself must be injected simultaneously with the whole bacilli, living or dead (130). Raffel (124) first showed that the "purified wax" of Anderson could substitute for whole bacilli. Choucroun (34) then showed that her lipopolysaccharide PMKo could also substitute for whole bacilli. Raffel (125) in a meticulous immunological study confirmed that "purified wax" could be replaced by one of its components, the previously described lipopolysaccharide. The use of killed mycobacteria as adjuvants for the establishment of the delayed type of hypersensitivity against various antigens has been generally applied and is reviewed by Freund (58). Thus, while hypersensitivity is demonstrably a response to protein within the bacillus, no convincing evidence has been offered that this hypersensitivity can be induced in the *normal* animal with the protein itself.

The Virulence of Tubercle Bacilli. "Cord Factor". Many observers have commented on the virulence of a young culture of tubercle bacilli compared with equal numbers of old cells. Bloch (15) noted differences in the character and distribution of the disease in mice infected with equal weights of three-day and three-week old cultures. Mice infected with the former died of miliary tuberculosis of lungs and heart; mice infected with the latter died of chronic tuberculosis. Middlebrook and Dubos (107) first noted "cords" in cultures of virulent strains and of BCG whereas avirulent strains formed irregular, piled masses. Bloch (15) extracted "cord factor" from virulent bacilli with petroleum; these cells remained viable but were avirulent and did not form cords. The extracted fraction inhibits leucocytes *in vitro*, and *repeated* doses are toxic to mice. The "cord factor" disappears from cells grown longer than about 14 days. Further information is awaited with eagerness.

IV. MODE OF ACTION OF THE TUBERCLE BACILLUS

We are now in the position to examine three important biochemical reactions conditioned by the host-parasite relation and to enquire further into the activity of the specific substances involved.

(a) Tubercle bacilli contain a lipin substance which enables them to reproduce and establish tubercles in the host. At present it is called "cord factor" or "virulence factor".

(b) The bodies of infected tubercle bacilli contain substances which resist degradation by the ordinary defensive mechanisms and are treated by the host in an unusual way. Instead of being engulfed by polymorphonuclear leucocytes and carried to the lymph nodes for digestion and elimination, they are absorbed *in situ* by monocytes which may subsequently be converted into a tubercle. It is noteworthy that this is the beginning of a usually successful self-limiting process, and it is tempting to regard it as a protective device on the part of the host. Numerous observers (102, 130) have been sufficiently impressed by the non-toxicity of multiplying virulent tubercle bacilli for normal tissue, or in tissue-culture preparations, to describe the association as symbiosis. Nevertheless, the immunity of the bacillus within the monocyte and, should multiplication ensue, the subsequent production of caseous tissue appear to indicate a common biochemical lesion associated with specific enzyme inhibition.

(c) The tubercle bacillus produces no pharmacological poison, either of exotoxic origin excreted during the life of the bacillus or of endotoxic nature liberated by lysis after its death. Should multiplication ensue, a product of metabolism induces hypersensitivity of adjacent host cells with the result that an otherwise innocuous product becomes a poison responsible for the death of cells. This remarkable host-parasite collaboration is responsible for most of the clinical manifestations of the disease.

(d) During infection the host may develop a capacity to modify the course of the disease—an acquired resistance.

The Specific Substances Involved. Biochemical lesion (a). "Cord factor" of Bloch (15) or "virulence factor" is a lipin mixture at present of unknown constitution.

Biochemical lesion (b). Chemiotaxis of monocytes (130, 102), as this has been called, seems to be a non-specific effect of long-chain fatty acids, of which the "phthioic acids" are examples, and of the products which contain them, the lipopolysaccharides (88).

Biochemical lesion (c). The hypersensitivity effect seems to be caused by tuberculin acting together with the lipopolysaccharide (130, 88, 85). Caseation seems to be caused by the inhibition of proteases by the lipopolysaccharide.

Biochemical lesion (d). The labile antigen which induces an acquired resistance is yet unidentified.

V. THE DIRECT ATTACK

Substances which Inhibit the Growth of Mycobacterium tuberculosis. With the reality of four or more effective chemotherapeutic remedies for tuberculosis in being, comes the prospect of the discovery of many effective substances. This justifies the devotion of rather more attention than is usual in reviews of this kind to the methods employed in the search. Let us first concern ourselves with the ways in which the activity of these substances have been defined.

Terminology: The Meaning of "Action". The vital nutrition of the micro-organism may be deranged on different metabolic planes. Should the enzymic processes

of respiration be affected, death is immediate; on the other hand, should less immediately vital systems be disorganised, cell division may become impossible. Alternatively, the metabolism of the cell may gradually come to a standstill because essential syntheses take unnatural directions. In this simplified view, the rate at which the vital processes are moving govern which end-point is recorded by the observer. Usually, the crude observation of the effect of poisons on micro-organisms has been that of sudden death (spoken of as a bactericidal action) or prevention of normal growth, or more usually multiplication (usually referred to as bacteriostasis). These traditional and imprecise terms have many shades of meaning. A number of observations on the mode of action of antibiotics have been described in these terms and are of limited value. Rahn's (127) comprehensive review may be quoted as an example of several which stress the essentially quantitative nature of estimates of bacteriostasis.

The action of many antibacterial substances may be modified or inhibited ("reversed") by other substances. In this way, the restoration of some or all of the biological functions of a bacterial cell is possible after the "death" of the cell, when judged by other standards. Many examples of reversibility have been noted and discussed elsewhere (22). It is sufficient for our purpose to note the antagonism between sulphonamides and p-aminobenzoic acid and the impairment of action of antibacterial metabolite-analogues by essential metabolites. A particular example from the general case of impairment of antibacterial action by ionic displacement (22), and germane to our immediate argument, concerns isoniazid. The addition of a cationic exchange resin to a culture of *M. tuberculosis* inhibited by isoniazid for 8 days allowed growth to be resumed and demonstrated the essentially inhibitory action of the drug (64). Our purpose is to emphasise the fact that unaided, the established antituberculosis agents do not kill the tubercle bacillus. On the one hand the host defence mechanisms are of overriding importance; on the other, the survival of the parasite is an implied hazard. Domagk (41) may be quoted in this context: "What are the special properties of these groups (streptomycin, *p*-aminosalicylic acid and the thiosemicarbazones) against the micro-organisms of tuberculosis? In the first instance it can be generally stated that none of them, even when applied for days or even weeks in concentrations much higher than those achieved in the diseased organism, leads to the killing off of the tubercle bacilli. What then is their effect? They inhibit the growth of the tubercle bacilli of all types. . .".

"*Protective Capsule*". The slow growth of the tubercle bacillus, its marked hydrophobic character and its survival in the host have raised a concept of a continuous protective lipoid capsule (130, 165). Yet, after a first isolation on an egg-enriched medium, freshly isolated tubercle bacilli grow on a simple medium containing glycerol as a carbon source, asparagine as a source of nitrogen, phosphates and a magnesium salt (50). Inexacting in its nutritional requirements, the *adapted* organism appears to restrict its growth factors to magnesium and phosphorus, which together with an alcohol and an aliphatic amino-acid (amide), water and oxygen, all readily diffusible water-soluble substances of poor lipin solubility, constitute its needs. There seems, however, to be little doubt that the

lipin-protein-carbohydrate complex constituting the cytoplasmic matrix is capable of resisting the passage of quite simple ions into the cell, since the tubercle bacillus maintains its internal environment in the presence of a broad range of acidity and basicity (139). Contact with 10% sulphuric acid for an indefinite period does not kill, 18% hydrochloric acid kills in 5 hours and 1% in 24 hours, while 5% acetic acid kills in less than 20 minutes (139). Equally impressive concentrations of bases are required to kill; 32% sodium hydroxide in 24 hours, or 40% in 4 hours. Barium and calcium hydroxide similarly are non-lethal (94).

Phospholipins inhibit the toxic action of many antiseptics on bacteria; for example, small amounts of cephalin protect Gram-positive bacteria against gramicidin *in vitro* and *in vivo*, and histones or protamines are able to combine chemically with active groupings of the lipoid complex of Gram-negative bacilli and thus render them susceptible to tyrothrycin or detergents which are otherwise inactive in these conditions (45).

In-Vitro *Tests.* All who have examined the evidence are impressed by the poor correlation between *in-vitro* tests for antiseptic activity and subsequent tests for chemotherapy. The reasons are discussed in recent reviews by Brownlee (21) and Feldman (49). Of the many reports which emphasise the unreliability of data obtained by *in-vitro* testing only three need be noted. Hogarth and Martin (73) selected 10 compounds with high *in-vitro* activity against strain H37Rv and tested the same compounds in mice. The drugs were absorbed yet none was active. The authors concluded "that *in-vitro* activity is a completely unreliable guide for the discovery of compounds with activity *in vivo*". A similar lack of relation was observed by Croshaw and Dickinson (36) who selected 24 compounds from a test total of 1000 compounds by *in-vitro* methods. Animal tests showed that none of the 24 was an active anti-tuberculosis drug. Meissner and Hesse (104) who reported testing 2000 substances by *in-vitro* methods had little success in relating *in-vitro* to *in-vivo* findings. It seems pertinent to reproduce once again the comment of Dubos (45) who listed the then few substances active in the animal and noted the limited, delicate, specific injury, directed, in those instances of which meagre information is available, against anabolic, synthetic processes, or steps in cellular division. In contrast, the bludgeon of antisepsis is directed to catabolic processes, or to anabolic and catabolic indiscriminately. It seems that while *in-vitro* procedures may have a place in an investigation of a closely related chemical series, the original member of which is a proved antituberculosis compound, it is unwise to rely upon the technique to explore new fields.

In-Vitro: In-Vivo *Tests.* The *in-vivo* failure of drugs effective *in-vitro* may be traced to many different causes. The most common are (a) the effects of metabolic processes, (b) poor absorption or rapid excretion and (c) toxicity. An attempt to meet these difficulties has been made by Brownlee (23, 21), Brownlee, Green and Woodbine (25) and by White and Karlson (72). A toxic amount of the test substance or the largest quantity that can be introduced is injected intraperitoneally in suitable suspension (23, 21, 25) or given orally (72) into a large guinea pig. After two hours, or before if toxic symptoms are seen, it is anaesthe-

tised and blood aseptically withdrawn by cardiac puncture. The citrated blood is used to establish an *in-vitro* series of graduated suspensions of virulent tubercle bacilli. A simultaneous comparison with a standard substance like streptomycin enables a reliable comparison to be made. Usually it is possible to determine the blood concentration microbiologically or chemically and in other instances the observation rests on the practical basis of a blood concentration which is optimum since it produces symptoms of acute toxicity or is derived from the maximum quantity it is possible to inject.

VI. IN-VIVO TESTS

Chick Embryo Tests. The chick embryo has been used in two ways to observe the effect of antagonists on tubercular infections. In one the chorio-allantoic membrane is sown; in the other, the bacilli are introduced directly into a small allantoic vein. Goodpasture and Anderson (62) described the invasion of the chorio-allantois by mycobacteria of both warm-blooded and cold-blooded type, and Moore (112) related the virulence of the pathogen to the nature of the tissue response. Emmart (47) applied the method to the study of the action of streptothricin and streptomycin. It seemed that 9-day old embryos were most suitable, and that a period of seven days after inoculation was satisfactory for observation. Gross tubercles were consistently produced on and in the chorio-allantoic membranes when virulent human bacilli were used. Emmart and Smith (48) believed the test to have advantages in estimating in a few days the virulence of a strain and also in showing activity against tubercle bacilli within six days. However Croshaw and Dickinson (36) found difficulty in devising a uniform infection, and the inconsistent results obtained by treating the infection with streptomycin caused these workers to abandon the method. It appears that the method of using the chorio-allantoic membrane of the chick embryo has not been generally accepted as a practical means of detecting substances active against tubercle bacilli.

The test in which 11-day-old chick embryos are injected intravenously with tubercle bacilli was described by Lee, Stavitsky and Lee (91, 90, 89). The same authors (91) described 840 quantitative estimations with blood from embryos treated with streptomycin, promin and sulphathiazole and detected appreciable quantities of these substances. Allantoic-amniotic fluid and yolk also contained significant amounts of drug. Of this test it may be said that the procedure is not simple and that the difficulties are many even for the expert. It cannot be claimed to be a practical method for large numbers of tests.

Rabbit Cornea Test. An interesting local test in which rabbits are used in that described by Gardiner, Rees and Robson (59). Anaesthetised mature rabbits are injected intracorneally in both eyes with a suspension of tubercle bacilli, and one eye is treated while the other serves as an untreated reference lesion. Drugs are introduced into the vitreous fluid at intervals during an experimental period of thirty days. Robson and his colleagues summarise the advantages of the test in the following way: (a) a dependable, progressive tuberculous process follows the inoculation; (b) the developing disease is visible and can be photo-

graphed; (c) since one eye only is treated, the untreated eye of the same animal serves as a convenient and dependable reference for comparison; and (d) few animals and small amounts of drug are needed to give reliable, preliminary information.

The effects of streptomycin and PAS used singly in intracorneal infections in rabbits compared favourably with results of the use of these drugs in guinea pigs and mice.

Mouse Cornea Test. The intracorneal method for establishing tuberculous infections has also been described for mice by Rees and Robson (129), with the difference that the drugs are given systemically and a control group of treated mice is used. Experiments with streptomycin, with PAS and with sulphetrone, three dissimilar chemicals, showed that tuberculous disease of the cornea of the mouse could be modified favourably by drugs of known efficiency.

The corneal test appears to have many advantages as a test giving reliable preliminary information. Compared with the rabbit test there is a saving in labour and time resulting from the administration of the drugs intravitreously two to three times weekly. Whether the test will prove sufficiently dependable for large-scale screening programmes will depend on additional development work. The preliminary reports are entirely acceptable.

Chick Test. Immature chicks infected intravenously with avian tubercle bacilli develop, in about ten days, typical tubercles especially in the liver and spleen. Two groups of workers, Solotorovsky *et al.* (153) and Carmichael and Maclay (31) have proposed tests based on this fact. Solotorovsky *et al.* treated infected chicks with streptomycin or dihydrostreptomycin for periods of six weeks beginning twenty-four hours after inoculation. They observed significant antitubercular activity within the drug-treated group compared with the control group.

In the method proposed by Carmichael and Maclay, therapy is delayed for three weeks. Groups of six chicks were treated with streptomycin alone, PAS alone and streptomycin and PAS given together. All exerted a favourable effect on the expected course of the disease. The observation that PAS alone was much inferior to streptomycin was an expected result in view of the inactivity of PAS *in vitro* against avian strains.

The advantages of the test are the use of an organism of low pathogenicity for man, and the observation of the effects of drugs on the pathogenesis of the avian tubercle bacillus in its natural host. Disadvantages are seen in the differences which exist between the avian pathogen and its natural host and the mammalian counterparts. A practical difficulty arises in providing disease-free stock and in housing growing birds for periods of about twelve weeks. Additional study is justified so that the value and limitations of the test may be fully appreciated.

Tests on Rabbits, Dogs and Monkeys. One outcome of the discovery of potent chemotherapeutic drugs is the study of their effects on the more intractable disease processes. It has long been recognised that the ulcerative pulmonary phthisis of adult man or reinfective pulmonary tuberculosis differs in its patho-

genesis from the miliary kind of blood-borne disease which claims kinship to the classical guinea-pig or mouse experimental infection. Rabbits, dogs and monkeys have all been used. Lurie (96) suggested the use of vaccinated genetically resistant rabbits infected by the quantitative inhalation of bovine tubercle bacilli to produce a localised phthisis. Francis, Spinks and Stewart (57) induced caseous ulcerative phthisis in dogs by intratracheal injection. After an initial lobar pneumonia there developed a chronic tuberculous process with "a striking resemblance to the phthisical lesions that may develop soon after primary tuberculosis infection in young human adults". The same authors (57) have described experimental tuberculosis in monkeys after the intranasal inoculation of human tubercule bacilli. The course of the infection and the nature of the lesions led the authors to conclude that tuberculosis in the monkey more closely resembles human tuberculosis than tuberculosis in any other animal studied by them.

These methods, in rabbits, dogs and monkeys, provide a means of observing the influence of drugs on intractable tuberculosis in which tissue changes are advanced and which is structurally unlike that induced in the guinea-pig and the mouse.

Mouse Test. The use of the mouse as an experimental animal for anti-tuberculosis chemotherapy emerged late, was quickly adopted once its advantages were appreciated, and has grown to impressive proportions. It is probably true to say that more tests with mice have now been made than with all other experimental animals put together. It remains to say that, its limitations recognised, the mouse test is probably the most useful single test available for examining large numbers of compounds.

The susceptibility of mice to tuberculosis had been lost sight of until Schwabacher and Wilson (143) described the course of the disease that followed the intraperitoneal or intravenous inoculation of very large numbers of tubercle bacilli. As a result of the reports of Youmans and McCarter (168) and of Martin (100) on the use of mice for testing anti-tuberculosis compounds, new interest was taken by many laboratories with the result that precise methods have arisen and exact techniques emerged. Although all strains of mice do not appear to be equally susceptible to infection with tubercle bacilli (120), the choice of a suitable inoculum appears to be decisive. The literature on the susceptibility of mice to tuberculosis is admirably reviewed by Raleigh and Youmans (128).

Choice of strain and inoculum. Human and bovine strains have been used with equal success (36, 129, 143, 120, 6). The high virulence of the selected strain should be perpetuated in the laboratory by an approved technique of subculture in a synthetic medium in which growth is slow. Most work appears to have been done with H37Rv, 'Ravenel' and D-4 (36, 129, 143, 120, 6, 44). Great variation in the size of inoculum is apparent in different reports. If young, dispersed growth cultures from the Tween-albumen liquid medium of Dubos are used (120), amounts as small as 0.001 mg. moist weight are successfully used by the intravenous route. Should older cultures prepared from solid media be used, amounts as large as 1.0 mg. moist weight may have to be used (100). It is well appreciated that grinding or even shaking a suspension of tubercle bacilli is a lethal procedure and it is supposed that old cultures prepared in this manner

contain many dead cells. A useful discussion relating the physiological age of the culture to its virulence has been contributed by Bloch (16).

Route of inoculation. The preferred method is to inoculate via the tail-vein, but respiratory (61), intranasal (44), oral (120), intracerebral (120), intraperitoneal (36, 120) and subcutaneous (44) routes have also been used. Mice inoculated intraperitoneally require about 10 times the intravenous inoculum for infection to occur.

Criterion of drug efficiency. Some authorities prefer to rely upon survival time only (6), but usually a more complete assay is attempted, including median survival time (ST_{50}), loss or gain of weight, and the nature and distribution of the tuberculosis.

Tests with Hamsters. Although it has been known for a quarter of a century that hamsters were susceptible to human and bovine tuberculosis it is only recently that the basic studies of Dennis and others (39) have shown them to be suitable for testing potential anti-tuberculosis compounds. Their report contains a useful review of the literature on the use of hamsters in experimental tuberculosis. The subcutaneous injection of 0.01 mg. moist weight of H37Rv grown in Tween-albumen liquid medium establishes a slowly progressive chronic disease with a mean survival time of 120 to 144 days.

The distribution of the disease in lung, spleen and kidney is similar to that seen in the guinea-pig, but the nature of the disease is totally different. Caseation, giant cells, encapsulation and calcification are usually absent. The distribution of the acid-fast bacilli is typically intracellular and the primary lesion a nodular epithelioid cell tubercle. Another and possibly related dissimilarity is the absence of skin allergy to tuberculin (39).

The effect of both streptomycin and PAS was to increase the expectation of life of infected hamsters but the bacteria were not eliminated so that death from tuberculous pneumonia occurred (39).

Elsewhere in this review is discussed the significance of the protection afforded to intracellular bacilli by the macrophages which engulf them. For example, bacilli ingested by rabbit macrophages are uninfluenced by PAS, are prevented from reproducing by streptomycin but die when treated with isoniazid (98).

The value of the hamster test would seem to lie less with the detection of novel substances having anti-tuberculosis action but with the more strict assessment of drugs of proved worth and, it may be, with the elucidation of their mechanisms of action. In this connection Feldman (49) suggests the merit of applying to the treatment of lepromatous leprosy any compound which eliminated intracellular tubercule bacilli in the hamster.

Guinea-pig Tests. The use of guinea-pigs for the assessment of potential new anti-tuberculosis drugs has been developed into a precise tool by Feldman and his colleagues who have described the method in detail (51, 81). Tests with guinea-pigs require much space, much drug and much time. Against these disadvantages are to be set substantial advantages. Treatment may be delayed until the disease is well established so that the value of any drug capable of promoting regression of the infection in an anatomical sense may be examined.

In these long term experiments the potential toxicity of would-be therapeutic

substances is also uncovered. The blood picture may also be controlled and blood drug concentrations studied. Since the guinea-pig shows a skin allergic reaction as one manifestation of a more general hypersensitivity phenomenon, this also provides a valuable, additional advantage.

The guinea-pig may be infected with a large inoculum of virulent bacteria to give a rapidly progressive fulminating infection or with a minute dose to give a slowly progressive but irreversible infection. By varying the amount and the route of the inoculum crucial information is obtained. For example, only the most potent anti-tuberculosis agent known to us will modify the infection in guinea-pigs inoculated intravenously with virulent tubercle bacilli and none will stay it (52). Animals inoculated with a large amount, 1 mg. moist weight of H37Rv, into the dorsal vein of the penis suffer a progressive infection of great rapidity, dying in 10 to 21 days. Treatment may not be delayed beyond four days and any prolongation of survival time indicates a more than ordinarily potent substance.

The most usual test is with animals infected subcutaneously with 0.1 mg. moist weight H37Rv and observed for periods of sixty days. They are then killed and examined for gross manifestations of tuberculosis, particularly in the spleen, liver and lungs. These organs may also be examined histologically for more precise information about the extent of the disease, and they may be cultured bacteriologically. A pattern emerges against which test is compared with standard and control. Drugs showing impressive evidence of modifying favourably the pathogenesis of tuberculosis may be examined critically.

A critical exacting test (52) is one in which the progressive, irreversible disease processes, induced by a small inoculum of 0.001 mg. moist weight, is allowed to become established before therapy is attempted. Treatment is continued, at the highest concentration of drug which is tolerated, for 150 days. The guinea-pigs are tuberculin-tested before being killed, and the control and treated groups are made the object of intensive histological and bacteriological studies. An important refinement is the inclusion of one treated group which is kept for observation until death. In this way, the ability of a drug to eliminate tubercle bacilli may be detected. To date no drug has fully met this latter requirement (49).

Criterion of drug efficiency. Of first significance in measuring drug activity is the extension of survival time and the absence of tubercle bacilli from the spleen. After a rather more effective drug, one would expect to observe signs of healing, as in the resolution of lesions demonstrable in histological preparations. These would range from the infiltration of epithelioid cells with fibrocytic elements to calcification and fibrosis.

Summary of Methods of Testing Anti-tuberculosis Agents. In-vitro *tests.* There appears to be general agreement that attempts to relate *in-vitro* results to *in-vivo* experiments have been disappointing. Authorities are disinclined to accept evidence of the action on cultures of tubercle bacilli in place of action against a tuberculous infection.

In-vitro:in-vivo *test.* This test has the virtue of ascribing tuberculostatic power only to the metabolised drug; also, acute toxic effects, if present, are seen, and

blood from test animals may be used to estimate blood-drug concentrations. Today, when more drugs of proven anti-tuberculosis activity are available, this test might well be critically re-examined.

Animal tests. No one familiar with the literature can fail to be impressed with the possibilities of the use of the mouse in distinguishing substances which suppress experimental tuberculosis in this animal. Its use in selecting substances having suppressive action in other animals is limited. It is to be expected that drugs will be found to be effective in mouse tuberculosis but not in the guinea-pig or man. However, used competently, with its limitations constantly in mind, the mouse-test seems destined to make a substantial contribution in the discovery of new anti-tuberculosis compounds.

The guinea-pig test is well established by virtue of the substantial amount of additional, confirmatory evidence it supplies. Similarly the more intractable experimental tuberculosis seen in the hamster, which may be associable with the intracellular distribution of the parasite, may give impetus to investigations with this animal in the future.

It is apparent that no single test can be accepted to the exclusion of all others if the essential confirmatory information, conservatively weighed, is to precede the clinical application of the drug.

VII. SPECIFIC ANTIBACTERIAL CHEMOTHERAPY

Of the biochemical lesions which follow the invasion by a tubercle bacillus, specific antibacterial chemotherapy is concerned only with the first, and even this is quickly modified to create the limitations associated with the use of our currently employed chemotherapeutic agents. The factors which influence the effect of chemotherapeutic agents on a bacterial population have been discussed elsewhere (20) and need only be listed. They are (1) the efficiency of the agent; (2) the effect of numbers of organisms; and (3) the rate of emergence of resistant strains. Corollaries to the first factor are (a) the inaccessibility of the bacilli in virtue of pathological changes, and (b) the inaccessibility of the metabolism of the bacillus in virtue of its metabolic inactivity. The latter two postulates have been discussed by Mackaness and Smith (98). The idea of a resting phase of the tubercle bacillus is hallowed by tradition (Koch, 84), honoured in time (Jensen, 78), and sustained by modern investigations (19). At first sight factors 1 and 2 appear to be related and indeed whether they are is dependent on the mode of action of the drug. For example, it is agreed that streptomycin is predominantly bacteriostatic; its activity varies with the concentration, the size of the inoculum, the medium and the pH. In these ways it resembles the sulphones, PAS and the thiosemicarbazones.

Many observers have shown that bacilli resistant to the inhibitory processes of known chemotherapeutic agents are present in any large bacterial population. The probability of resistant-organisms being present therefore depends on the size of the population. As organisms resistant to one agent are not necessarily the same as those resistant to another, the exhibition of one additional inhibitory substance will reduce the resistant population. There is experimental evidence

that limitation of numbers has, indeed, reduced the rate of emergence of resistant strains (142). Much of the evidence for the mutation theory of emergency of resistant strains is provided by the statistical methods developed by Luria and Delbrück (87); those interested should consult a recent critical review by Cavalli (32).

Hypersensitivity Effect. One other biochemical lesion is actively concerned with the spread of the disease. This is the hypersensitivity phenomenon, recently discussed by Brownlee (20). An analogy has been drawn with the phenomenon of histamine sensitisation and its modification by antihistamine drugs. It is believed that specific drugs will be found to block the delayed anaphylactic reaction to tuberculin conditioned by the lipopolysaccharide. The same author also describes, again by analogy with histamine, successful attempts to desensitise man with tubercle vaccine and tuberculin.

Acquired Resistance. While infection with tubercle bacilli does not confer the stable and solid protection acquired after diphtheria or smallpox, there is little doubt that a significant degree of protection is attained (130). Although widespread agreement exists that the living attenuated bovine bacillus of Calmette, BCG (30), confers recognisable acquired resistance in laboratory animals, there has been more reluctance to accept the claim for killed vaccine. A meticulous and critical reviewer like Rich (130), writing in 1944, speaks of the established fact doubted for years, and quotes numerous successful investigators, yet Raffel (126) is unable to satisfy himself that a heat-killed vaccine confers resistance. More recently there have been comparisons made between BCG and vaccines killed by irradiation with ultraviolet light in which the vaccines were given by intraperitoneal injection on a number of occasions before challenge in guinea-pigs (116, 140) and in mice (109). In these three instances significant protection was claimed for the irradiated vaccine. Given as a single vaccination, intracutaneously, intravenously or intramuscularly in the guinea-pig, BCG gave significant protection against challenge by tubercle bacilli, whereas irradiated vaccine gave none in the hands of Feldman and his colleagues (144). We may infer from these apparently contradictory observations that BCG given in a single vaccination confers good protection in the guinea-pig and perhaps in the mouse; that killed vaccine under similar conditions of dosage confers little or no protection; that given in a number of doses previous to challenge a suitably prepared killed vaccine confers some protection but this does not approach that of BCG. Brownlee and Kennedy reported (21) that the glycerol killed vaccine of Griffith and Glover (61a) conferred a significant degree of protection, but less than BCG in the guinea-pig. Their animals were vaccinated intramuscularly, on four occasions at intervals of ten days and challenged by infection at six and a half weeks after the first injection. The disease was slow and chronic, terminating 44 weeks after injection.

That the choice of experimental animal and the nature of its tissue response to *Mycobacterium tuberculosis* are important is illustrated by the effect of BCG in the hamster. Houduroy (74) reports that a BCG ampoule taken at random and injected into the hamster caused its death one year later. A three-week-old cul-

ture of acid-fast organisms from the spleen was non-toxic to the guinea-pig or rabbit but killed hamsters.

Attempts to demonstrate acquired resistance with BCG and killed vaccine as in the laboratory animal have probably also been successful for man (76, 83). However, no recombination of the inadequately known chemical fragments has yet proved successful. It seems that the antigenic complex which confers resistance is labile and readily destroyed by chemical manipulation. Its demonstration may await the application of less unphysiological methods like extraction of the living cells with glycerol or urea solutions, perhaps at low temperatures. The direct synthetic approach may be expected, in this instance, to make a helpful contribution to our knowledge of that particular biochemical lesion which is identified with chemiotaxis of monocytes, with inhibition of proteases, and perhaps with inhibition of β-oxidation. One such substance is 3:13:19-trimethyltricosanoic acid (132). This was once thought to be phthioic acid, but the latter is now believed to be an α,β unsaturated acid (33). Of great interest are additional synthetic analogues of enhanced biological activity (164). The elegant anodic syntheses of long-chain fatty acids of known isomeric configuration by Linstead and his collaborators (93) may be expected to forward this vexed problem. The description of the lipin-bound polysaccharide, which is a hapten of high specificity, by Haworth, Kent and Stacey (68) and the isolation of pure crystalline protein (tuberculin) of Seibert, which is a known antigen (146), carry the attack forward.

Artificial Antigens. For a description of the unsuccessful attempts to prepare artificial protective antigens to experimental challenge by tubercle bacilli, a previous review should be consulted (21).

VIII. CHEMOTHERAPEUTIC SCREENING

In 1932 Wells and Long (165) assembled the existing knowledge of tuberculosis and concluded that no known remedy modified the disease in the experimental animal or man. When the subject was reviewed in 1948 (21) Domagk's very great discovery (42) of "prontosil rubrum", active against experimental infections due to virulent streptococci, had provided a new impetus and the anti-tuberculosis activity of diaminodiphenylsulphone and its derivatives was known. Also reviewed at that time were the chemotherapeutic studies with streptomycin and *para*-aminosalicylic acid (PAS). Reviewed in 1951 (41), the list had grown by the addition of the effective but toxic thiosemicarbazones introduced by Domagk and his colleagues (43). With the persistence which had characterised his earlier discovery Domagk followed the faint trail of inhibition of the growth of the tubercle bacillus by sulphathiazole to a sulphathiadiazole compound and then to the substituted benzaldehyde thiosemicarbazones. There is little doubt that the simultaneous discovereis (13, 158, 133) in a number of laboratories of the marked chemotherapeutic properties of the hydrazine derivatives of isonicotinic acid is to be directly traced to the development of the thiosemicarbazones.

The earliest reports (13, 158) of the anti-tuberculosis activity of isonicotinic

acid hydrazide (isoniazid) made it clear that the substance produced its effect in doses lower than any synthetic or natural compound hitherto tested. Variations tested to date have yielded no more effective compounds (14). In mice its minimal effective dose is $\frac{1}{100}$ that of PAS (13); it protects the tuberculous guinea-pig and rabbit (158). In man there is an impressive therapeutic effect in acute caseous-pneumonic tuberculosis (133); for this purpose it compares in efficiency with the combination of streptomycin and PAS (113). When the drug is used as the sole therapy in man, resistant strains emerge rapidly (113), 11% being observed after one month, 52% after two, and 71% after three months. Moreover, lack of clinical progress seems to be related to the rate of emergence of resistant strains. Later therapeutic trials (114) appear to show that the rate of emergence of resistant strains is low when isoniazid is combined in treatment with streptomycin and PAS. Indeed, after three months of therapy, it appears to be less than 1% (114).

The general problem of the emergence of resistant strains is discussed elsewhere in this review and mention need only be made here of aspects peculiar to isoniazid. One of the first reports (158) of the anti-tuberculosis properties of isoniazid noted that a delayed growth of tubercle bacilli appeared after five weeks in a Tween albumen medium containing 25 μg. per ml. of drug. These organisms were shown to have an increased resistance to isoniazid. A similar observation based on five strains of *Mycobacterium tuberculosis* grown on a solid medium was made by Hobby and Lenert (70) who demonstrated the presence of cells resistant to at least 50 μg. per ml., a 1,000-fold increase, in cultures observed for 12 days in liquid medium containing isoniazid. They interpreted this to mean that large numbers of resistant cells were present among the initially large population of sensitive cells. They made the curious observation "that resistance can be demonstrated in this manner only at times and that the same strains, while tested for their sensitivity to isonicotinic acid hydrazide on repeated occasions, often will show no evidence of cells resistant to this antimicrobial agent". The experiments of Fisher (55) may prove to have shed light on this interesting observation. She found that the strain H37Rv (included in Hobby and Lenert's series), when made not less than 5- to 100-fold resistant to isoniazid, had lost its ability to grow in the synthetic medium of Dubos and Davis but grew normally when additional amounts of bovine albumen (fraction V) or bovine serum was added. As the resistance to isoniazid increased, so did the need for additional albumen. No known vitamin or aminoacid could replace it. Contrary to the claim of Pansy, Stander and Donovick (118), isoniazid resistance is not easily lost, *id est*, the resistant mutants *grow* when their growth requirements are met. An obvious implication of this work is that the isolation and identification of strains resistant to isoniazid should be attempted only in medium proved to support their growth.

Isoniazid: Mode of Action. A beginning has been made with experiments on the mode of action of isoniazid. The bacteriostatic action has been interpreted (7) in terms of a depression of an unknown growth factor; Yorida, Kato and Okijima (174) believe its action on the formation of indole by *E. coli* to be consistent with

the view that the drug acts as an antimetabolite against the coenzyme, pyridoxal phosphate.

Streptomycin: Mode of Action. Umbreit and his colleagues (161, 115, 162) have shown that streptomycin blocks the terminal oxalacetate-pyruvate condensation essential to the oxidation energy processes of *E. coli*. Resistant cells do not have the oxalacetate-pyruvate condensation system enzyme (151). Bacteria are inhibited within the host without harming the latter because streptomycin does not penetrate to the site of this reaction in the animal cell (163). Is the reaction under discussion that which results in the death of the streptomycin-sensitive cells? All that can be said is that streptomycin, dihydrostreptomycin and mannisidostreptomycin—all antibiotics which inhibit growth—also inhibit this reaction. Inactive streptomycin derivatives do not. No evidence for an oxalacetate-pyruvate condensation system has been uncovered for *Mycobacterium tuberculosis*, but an analogous mechanism involving the oxidation of fatty acid has been implicated by Umbreit (162).

Other Chemotherapeutic Antibiotics. Nisin. The earlier claims for the chemotherapeutic action of nisin against experimental tuberculosis in the guinea-pig (101) have been challenged by the failure to protect the rabbit against experimental bovine tuberculosis (63). *Cepharanthine.* Despite the continued clinical claims that this alkaloid modifies favourably the course of phthisis (67), the only satisfactory experimental evidence is of its failure to influence the experimental disease in the guinea-pig (21) and its failure to modify the course of rat leprosy (141). *Myomycin*, isolated by Johnson and Burdon (80), suppresses experimental tuberculosis in mice (77) but is limited in its application by instability at normal temperatures. *Neomycin*, isolated by Waksman and Lechevalier (169), was shown by Feldman and his colleagues to have significant anti-tuberculosis action in guinea-pigs (82). The neomycin complex described by Waksman and Lechevalier has now been shown to contain at least four antibiotic substances, named neomycins A, B and C and fradicin (66). Tested simultaneously against a strain of bovine tubercle bacilli (BCG), the following antibiotics inhibited reproduction at a concentration given in μg. per ml., neomycin B, 0.9; neomycin C, 2.6; streptomycin, 0.5; chloramphenicol, 1.25; aureomycin, 0.15 and streptothricin, 2.8 (66). Only the misleading nature of the information need be commented on at this stage; neither aureomycin nor chloramphenicol significantly modifies experimental or clinical tuberculosis. *Catenulin* is said to be similar to the neomycins from which it may be distinguished chromatographically. Neurotoxic and cumulative intoxications have been seen in cats (38).

Terramycin. The Pfizer group of workers described this wide-range antibiotic with properties similar to aureomycin (71, 157). Its capacity to suppress experimental tuberculosis infection in guinea-pigs and mice and its low toxicity in man justified its trial in phthisis (54); for this purpose its value seems limited. However, combined terramycin and streptomycin therapy for pulmonary tuberculosis appears to provide chemotherapy as effective as any combination hitherto described, with the advantage that strains resistant to either drug may not emerge (108). In the interesting test of Mackaness (97) previously discussed, by which

the capacity of drugs to penetrate and inhibit the growth and reproduction of tubercle bacilli phagocytised by rabbit macrophages is observed, terramycin was found to be as inhibitory to intracellular growth as to extracellular growth of the bacilli. The strain of bovine tubercle bacilli was inhibited by a concentration in the medium of 12.5 μg. per ml. This faculty of inhibiting intracellular acid-fast organisms, suggests the possibility that terramycin might intervene in lepromatous leprosy. Terramycin and aureomycin appear to have a similar mode of action in their ability specifically to inhibit the "acid-soluble organic phosphorus fraction" of the phosphorylation system of bacterial metabolism (110).

Viomycin. Hobby and her colleagues (72) described the anti-tuberculous activity of viomycin, and McDermott and others (166) reported a clinical trial, limited to 10 persons with advanced pulmonary tuberculosis treated for 182 days. Of these, renal irritation and abnormal serum electrolytes were observed in five, disturbance of vestibular function and deafness in two, and hypersensitivity in three. There was a limited increase in strain resistance to viomycin in 60 days. The status of this interesting antibiotic is still under examination but it would appear that its range of efficiency is limited and its toxicity sufficient to warrant care in its use at the dose level employed in the study cited. *Erythromycin* has been described as an antibiotic of broad range but this seems a little generous. It is claimed to have anti-tuberculosis activity in mice and is of low toxicity for mice and dogs (103).

Antibiotics In Vitro. Three new antibiotics, all derived from *Streptomyces* sp., have been described. *Mycobacidin*, which inhibits various acid-fast bacilli including tubercle bacilli at 10 μg. per ml., is interesting because of a low acute toxicity when given intramuscularly at 1 gm. per kg. (159). *Thiolutin*, taking its name from the brilliant yellow colour, is sparingly soluble in water, inhibits various mycobacteria including tubercle bacilli at 5 μg. per ml. and has an oral LD_{50} for mice of 25 mg. per kg. (149). *Cinnamycin*, a polypeptide characterised by the presence of sulphide aminoacids, inhibits strain H37Rv at 5 μg. per ml. (11). *Globicin*, extracted from *Bacillus subtilis*, is claimed to inhibit avian tubercle bacilli at 10 μg. per ml. (123).

Those who are interested in the anti-tubercle bacilli action of the juices of higher plants should consult an excellent account by Azarowicz, Hughes and Perkins (5) which also contains a good bibliography.

Additional Chemotherapeutic Claims. For a list of references to additional claims for chemotherapeutic activity in the experimental animal, a previous review should be consulted (21).

Empirical In-Vitro *Screening.* A survey of the literature of *in-vitro* experiments since the assessment of Wells and Long (165) can convey but a hint of the field covered since every student of the subject is aware of the existence of a huge total of additional examinations made within commercial organisations. The purely negative results are seldom published. A survey of "potential tuberculotherapeutic compounds" by Guss and Kloetzel brought the list up to date in 1948 (65). Thereafter the most complete survey is to be found in the subject index of "Chemical Abstracts" in which *in-vitro* effects are listed under *Myco-*

bacterium tuberculosis" and effects on animals under "Tuberculosis". The reviewer made the following comment in 1948 (21), "It is a task of dubious significance to identify the possible leads indicated by the enormous total of *in-vitro* experiments in which acid-fast bacilli and tubercle bacilli of various origin, known and unknown, have failed to grow in the presence of added substances. No criticism of the many admirable detailed studies of variations of activity within a chemical series, as such, should be read into these remarks; however even here the severe limitations of *in-vitro* comparisons usually appreciated at their source, are often lost to the unsuspecting into whose hands fall the surveys of 'anti-tubercular drugs' ". Today the position is much improved, and it is becoming increasingly rare for claims for *in-vitro* experiments against tubercle bacilli, unsupported by anti-tuberculosis animal experiments, to be accepted by editors as adequate research information. However, in the following section apparently significant leads derived from *in-vitro* tests will be discussed.

Growth Requirements in Mycobacterial Species. Three pathogenic organisms, Johné's bacillus (*Mycobacterium paratuberculosis*), Koch's bacillus (*Mycobacterium tuberculosis* var *hominis*) and Hansen's bacillus (*Mycobacterium leprae*), together with the non-pathogenic timothy-grass bacillus *Mycobacterium phlei*, form a closely related acid-fast group. In 1911 Twort and Ingram (160) demonstrated, probably for the first time for any micro-organism, a clear growth-factor requirement for Johné's bacillus. The growth factor(s) was supplied by vaccines of other acid-fast bacteria including *Mycobacterium tuberculosis* var *hominis* and *Mycobacterium phlei* and it was found that lipin solvents could extract the principle. For some time it was believed that phthiocol (3-hydroxy-2-methyl-1:4-naphthoquinone) isolated from tubercle bacilli, and synthetic 2-methyl-1:4-naphthoquinone, a biologically active vitamin K analogue, were growth factors for Johné's bacillus (173) but this is now known to be untrue (56). Subsequently there was reported the isolation in the form of a crystalline aluminium derivative of an approximate formula $C_{49}H_{75}O_{10}N_6Al$; it is apparently not a naphthoquinone. A number of inhibitors modelled on vitamin K-active naphthoquinones have been found to inhibit the growth of tubercle bacilli in the test-tube but these and similar analogues have proved inactive in the experimental animal (21).

Usnic acid is a naturally occurring quinone which is of potential activity *in-vitro* against the tubercle bacillus (28). It has been shown to protect the tuberculous guinea-pig, and to act synergistically with streptomycin (99), with which it forms a usnate called Usnemycin (28), and to be active in cutaneous tuberculosis (119).

We have discussed elsewhere (21) the contribution of Barry and his colleagues to the chemistry and anti-tubercle bacilli activities of a series which had its origin in the substance called diplocin extracted from the lichen *Buellia canescens*. These are phenazine compounds, one of which is of interest; it has been called B283. It has since been found to have anti-tuberculosis activity in mice and guinea-pigs and to have a favourable influence on lepromatous leprosy (8).

Starting from roccellic acid, which is methyl-dodecylsuccinic acid, Barry and others (8) encountered maximum *in-vitro* activity against tubercle bacilli at a

chain-length of 13–15 carbons. A half ester of heptylsuccinic acid was the most active in the test-tube. This substance protects the tuberculous guinea-pig to a limited degree and, like the usnic acid types, has been used in the management of local tuberculous lesions (8).

Surface-active Anti-tuberculosis Compounds. A significant discovery likely to have far-reaching effects has been recently reported by Hart and his colleagues (35). Others observed that the surface-active substance known as 'Triton A20', which had been used as a substance which promoted the dispersed growth of virulent tubercle bacilli, also kept the blood concentrations of cholesterol and phospholipids of experimental animals at a high level for several weeks at a time after intravenous injections. Hart *et al.* (35) interested themselves in this observation as a factor likely to influence the susceptibility of mice to experimental tuberculosis. But they made the surprising discovery that 'Triton A20' caused a significant suppressive effect on the course of a moderately acute tuberculous infection in mice. This observation has been amply confirmed. 'Triton A20' is an aqueous solution of an arylalkyl polyether of phenol. Variations in the octylphenol units within a series synthesised and examined by these workers showed more protection to lie with molecules of higher molecular weight. It is of great interest that these compounds have negligible bacteriostatic activity.

Several aspects of the protection given by these compounds is worthy of note. First, it does not appear necessary to administer the drug continuously, as is done with streptomycin. Again, unlike the antibacterial chemotherapeutic drugs, its duration of protection extends considerably beyond the period of administration. To quote the observers, "When, however, the type of experiment was varied by killing about eight (instead of three) weeks after cessation of treatment most of the streptomycin-treated mice had died of chronic pulmonary tuberculosis whereas the animals treated with 'Triton A20' (15 mg) were still alive although their lungs showed that the infection had progressed". Subsequently Hart reported (37) that a guinea-pig test had showed protection and healing comparable with that in a streptomycin group. An *in-vivo*:*in-vitro* test had been negative with blood taken from heavily treated animals, which confirmed the fact that 'Triton A20' was not functioning as a classically bacteriostatic agent. Also he observed that chemotherapeutically active members of this series of surface-active compounds also depressed the tuberculin sensitivity in BCG-immunised guinea-pigs.

The importance of these observations can scarcely be overrated. Without attacking the tubercle bacillus directly, these substances interfere with the production of tubercles. Just which of the mechanisms already discussed in this review, either that involving the lipopolysaccharide or tuberculin, or both, is involved is a fit subject for speculation.

Ali-esterase Inhibitors of Tubercle Bacilli. Mendel and others (106) have recently shown that the growth of human tubercle bacilli in a Dubos medium is inhibited by diethyl p-nitrophenyl phosphate (E 600) in a concentration of 0.5 μg. per ml., and by di-isopropyl fluorophosphate (DFP) at twenty times this concentration. These cholinesterase inhibitors are powerful inhibitors of ali-esterase ac-

tivity (the enzyme which splits aliphatic esters like triacetin and tributyrin), and interest is aroused because of the relation between inhibition of growth and the degree of ali-esterase inhibition.

IX. EPILOGUE

"Cet animal est très méchant
Quant on l'attaque il se défend".

This animal is very mischievous; when it is attacked it defends itself.

La ménagerie, by Théodore P. K., 1868

The discussion of material in this review gains impetus from new knowledge about the tubercle bacillus secured in the last decade,—about its chemistry, its metabolism and its chemotherapy. The successful application of chemotherapy to the field of allied bacterial disease, which Wells and Long prophetically expected to inspire the more difficult attack on the tubercle bacillus, has provided an effective stimulus. We may confidently expect more effective antibiotic agents to be discovered and more effective chemotherapeutic agents to be synthesised; in objective the empiric approach has become routine; only the methods differ.

The object of the direct approach to the chemotherapy of tuberculosis has been stated simply as "the elimination of the parasite". No niceties of definition should be allowed to obscure the facts about the inability of the currently successfully used anti-tuberculosis drugs to *kill*; their action is but inhibitory; inherent, then, is the cooperation of the host. Let us leave the discussion of the influence of drugs on the tubercle bacillus with a question. Is the incidence of *susceptible* survivors a direct result of the use of inhibitory drugs?

Of the capacity to defend itself enjoyed by the tubercle bacillus we shall content ourselves with a comment on three aspects. The pathological changes induced by the organism constitute a mechanical hazard to penetration by the attacking chemical substance. Playing a large part in these changes is the hypersensitivity phenomenon which seems to cause the death of the host cells. The "chemical" substances implicated in this biochemical lesion are tuberculin and the lipopolysaccharides. May we not believe that pharmacologically active blocking agents may yet be found to block this reaction? The slow metabolism of the causal parasite, which in unfavourable circumstances is so reduced that metabolic drug-competition is ineffective in that substances hitherto effective no longer constitute a hazard to the continued 'life' of the bacillus, endows it with a notable capacity to survive. Finally there is the statistical risk arising from large bacterial populations; if large enough there is found to exist a number of mutants unaffected by the attack of any single anti-tuberculosis substance. Research in this field has been more than ordinarily fruitful and is likely to continue so to be.

The indirect approaches derive their sources from the purposeful studies of the biochemical lesions caused by the chemical fragments of the heat-killed organism, lipin, protein and carbohydrate. An appreciation of the degradatory nature of these substances prompted a search for more physiological molecules. Of these

the lipopolysaccharide fraction has marked potentialities. It is to be hoped that experimental evidence may now uncover its mechanism of action in attracting monocytes and in inhibiting their enzymes. Of its part, together with tuberculin, in sensitizing host cells so that they die, much will be heard in the future. The search for pharmacological blocking agents, now begun, is an urgent problem.

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