THE BACTERIOLYTIC ACTION OF GLAND EXTRACTS ON TUBERCLE BACILLI.

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The extent to which animal tissues are capable of destroying tubercle bacilli is of great importance, because of the heavy toll of lives which tuberculosis yearly demands, because of the peculiarly resistant nature of the bacillus itself, and the very questionable immunity which it is capable of producing.

The degree of immunity existing in tuberculosis is unknown. The bacillus, like other organisms, contains specific protein, which when inoculated into the animal body is capable of giving rise to protein antibodies, such as precipitins and agglutinins. These were to be expected. Just how far these protein re-actions are an expression of a truer immunity is difficult to say; they may well be regarded as by-products of immunity which have little significance as regards the resistance of the body to infection. Even if the occurrence of tubercle protein in the bloodstream be invariably followed by the advent of antibody, there is no proof that tubercle toxin will call forth antitoxin in the ordinary sense of the term. Tuberculous serum may be toxic, but it has not been found antitoxic. Again, tubercle bacilli may circulate in the blood, but no production of bacteriolysin will follow. Arloing has found that tubercle bacilli, agglutinated by an antiserum, appear to grow even better than if the serum had not been present. The only blood which has been described as bacteriolytic to tubercle is that of *Galeria mellonella*. Sieber and Metalnikoff, who observed this bacteriolysis, attributed it to a somewhat heat resistant ferment, and not to the ordinary mechanism of complement and amboceptor. Treatment with bacterial products may indeed be followed by an increase in the phagocytic power of the blood, but in how far this property may be regarded as advantageous depends entirely on the fate of the bacilli after ingestion. Living bacilli, carried in the bloodstream

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by leucocytes, might prove a source of danger rather than an evidence of a means of defence. Leucocytes are, however, known to be peculiarly rich in ferments, and dissolution of many species of bacteria ingested does take place, provided that the particular ferments suited to the bacteria are present.

Power of Tissues against Tubercle.

Tuberculous tissues do appear to exert some destructive influence on the bacilli *in vitro* and *in vivo*. Much has described partially degenerate bacilli in lupus, known as "Much's forms," which have lost their acid-fast properties. Fontes, who also found these forms in tuberculous pus, experimented with extracts of caseous glands on living bacilli. He found that the numbers of the bacilli were thereby reduced, the bacteriolytic substance remaining active for 120 days. Wolff has found Much's forms in the glands of otherwise non-tuberculous children, *post mortem*. Of all human tissues, the skin appears to exert the most remarkable influence on tubercle bacilli growing in it. Not only can it rob the bacilli of their characteristic staining properties, but, as the careful work of Griffiths has so clearly shewn, the virulence of the bacillus is profoundly altered. Lupoid material injected into guinea-pigs has sometimes given rise to no lesions whatever, although the living, but non-pathogenic bacilli could be afterwards recovered and cultivated from glands and omentum. Guinea-pigs treated by Spiro with extracts of tuberculous lymphatic glands were rendered thereby more resistant to a later experimental tuberculosis. Trudeau, in similar experiments, obtained partly favourable results with human tuberculous gland preparations. There is also the work of Kitasato in 1892, proving that the majority of bacilli in sputum, though staining well, are already dead.

That normal tissues can exert an anti-tuberculous influence has also been demonstrated. Markl has recorded a complete bacteriolysis of tubercle bacilli both within and outside phagocytes in peritoneal exudates in guinea-pigs which he examined at intervals within 24 hours after intraperitoneal injection of the bacilli. Kling has seen some bacteriolysis of tubercle bacilli when these were treated with leucocytic extracts *in vitro*. Calmette and Guerin lowered the virulence of the bacilli by means of bovine bile, as did Bartel, Neumann, and Leimsner by using proteolytic ferments, lipoids, and fatty soaps extracted from the spleen, liver and lymphatic glands. Neumann and Wittgenstein

found that in normal lymphatic glands, pancreas, liver, or ovary, the virulence of tubercle bacilli was either entirely lost, or at least so much lowered that the bacilli after intravenous injection were no longer able to cause generalised but only local infections. Defibrinated blood, or lung tissue, had no such influence. Wittgenstein also found that the life of tuberculous animals could be prolonged by treatment with ovarian substance. Schroeder found that experimental tuberculosis ran a more chronic course in guinea-pigs treated with spleen substance. Withe and Zeublin investigated the influence of some fresh and autolysed gland extracts on the virulence of tubercle bacilli, and found that autolysed extracts of rabbit's lung and liver exerted a marked influence. Unfortunately they do not record the exact acidity of the autolysed extracts, although they mention that the liver extracts were very acid. These authors did not regard any bacteriolysis as having taken place, as the bacilli were unchanged in form, and staining properties. Deycke and Much have described a complete dissolution of tubercle bacilli in certain brain substances, such as cholin, neurin, and lecithin, which has been attributed by Tessen and Rabinowitsch to alkalinity alone. Sieber and Metalnikoff have found some strains of tubercle bacilli actually dissolved by lipase. Perhaps the best, because the best known, demonstration of anti-tuberculous power on the part of the tissues is afforded by the selective tendency so characteristic of tuberculous infection. One species is preferred to another species; one organ to another organ. Take, for example, the comparative immunity enjoyed by the rabbit's liver in comparison with its highly susceptible lungs, or the remarkable rarity of tuberculosis in the sheep in comparison with its frequency in the ox.

NATURE OF THE BACILLUS.

The highly resistant qualities of the tubercle bacillus, its slow growth, and its acid-fast properties are believed to be due to its envelope, which contains a high percentage of fats and waxes, and which is only penetrable with difficulty by bacteriolytic substances, food material and solvents. If this fatty envelope could be first dealt with, the bacillus itself would present less difficulty. This fatty substance has been shewn to consist in great measure of fatty acid esters of glycerine and especially of higher alcohols. Baudran found the percentage of fatty material varying in different strains from 36 % to 44 %. Kresling extracted 38·95 % of total "fat" from the bacillus, of which 39·1 %

was present in the form of fatty acid esters of higher alcohols alone. With the lipases which the animal body contains and by means of which it splits esters, it might be possible to break down the envelope and reach the bacillus. A bacteriolysis of the tubercle bacillus might be effected through esterases in this way, either by physical disruption, by increasing solubility, or by the production of acid.

Lipolysis and Bacteriolysis.

The two methods of destroying bacilli which most readily suggest themselves as within the scope of the defences of the body, where heat is in the case of the pathogenic organisms hardly applicable, are certainly on the one hand the production of acid or alkali, on the other hand solution of some constituent of the bacillary wall. Take, for example, the destruction of the colon bacillus in bladder infections when the urine becomes alkaline, or the effect of lactic acid in sour milk on the organisms of the intestine, or again the action of the acid stomach contents upon cholera spirilla. Many antiseptics are solvents, especially fat solvents; for example, alcohol, ether, carbolic acid. In ferment action, more particularly proteolytic and amylolytic action, the body has the power of promoting solubility. This is not so much the case in lipolysis, where the end products, fatty acid and alcohol, are often both highly insoluble. These higher alcohols, notably cholesterin, are, however, either soluble or at least emulsifyable in the fatty acids themselves. The action of free fatty acids upon these alcohols is very remarkable. Although no ester has formed as can be proved by titration, a curious physical change takes place. Add solid fatty acid to warm water in which insoluble cholesterin crystals are lying, the water becomes milky and the cholesterin crystals disappear. Even solid fatty acid falling down in cold water with a clear supernatant fluid, will, if in small pieces and shaken, be taken up into a milky emulsion on the addition of tubercle bacilli, which contain free alcohol. It is interesting that Cramer, Feiss, and Bullock find that "mixtures" of fatty acid and cholesterin stain differently to their esters also differently to either alone. Whether such mixtures represent a solution of the alcohol in the acid, or a loose chemical bond, the ester gives firm union, insolubility and impenetrability; the mixture, from its emulsion-forming tendencies, rather gives penetrability and fine division.

The body has much power of actually dissolving bacteria other than tubercle; both leucocytes and the body fluids have been proved

to possess this power. The actual dissolution of acid-fast bacilli is not easy to observe in body fluids, but it has been abundantly proved that though actual dissolution is so difficult and slow, bacilli have been killed by the tissues even while remaining perfect in form and staining properties. Acid-fast bacilli killed by acid *in vitro* are apparently unchanged in form and staining properties. Lipolysis is accompanied by the production of acid, which may be a low, soluble fatty acid, or a high, insoluble one. Lipolysis requires not only ferment and fat but also an activating agent. Activation is believed to depend upon a removal of the inhibitory products of ferment action. Thus $CaCl_2$, recommended by Kanitz, has been shewn by Pekelharing to act by forming insoluble soaps of calcium, and so by precipitation to remove the products which are hindering further action. The action of bile salts is quite opposite to this; by promoting solubility, especially in the duodenum, they get rid of the end products of lipolysis by their absorption through membrane. This suggests that should fatty acids be absorbed by tubercle, no other activator would be required in the lipolysis of the fats surrounding the bacillus. The milky emulsion just described which takes place when tubercle bacilli are shaken in water with insoluble fatty acids demonstrates that tubercle bacilli can take up these acids. In another communication (Porter, *This Journal*, XVI. p. 66) on the sensitiveness of tubercle bacilli to acid, I have shewn that bacilli are actually killed by these higher fatty acids which though insoluble in the watery medium round the bacillus are yet able to penetrate its fatty envelope when applied to it. If this be so, how much more easily could the bacillus be killed by fatty acids formed in the envelope itself during lipolysis, even though its form and staining reactions remain unchanged. Moreover a preliminary lipolysis may give access to proteolytic ferments which produce also soluble fatty acids, and have greater solvent properties.

With these considerations in view, I was led to study more fully the distribution of lipases, and to examine a number of organs for wax-splitting power, on account of the presumably great importance of these ferments to the resistance of the body against tuberculosis. The results of this investigation have been published elsewhere (*Biochemical Journal*, 1916, vol. x. p. 523) but are briefly indicated in the following tables for purposes of comparison.

As lipolysis is accompanied by acid production it was necessary to estimate the sensitiveness to acid, inorganic and fatty, of the bacilli under investigation. It was found (*This Journal*, XVI. p. 66) that tubercle

bacilli were killed in the presence of $n/10$ acid, but could resist weaker concentrations of acid for 24 hours, while the other acid-fast bacilli could not resist $n/80$.

Extracts of the organs tested for esterases, have also been examined for bactericidal power against tubercle bacilli.

Methods.

Extracts were made from the following organs:

	Human	Ox	Sheep	Pig	Cat	Rabbit	Guinea pig
Pancreas	×	×	×	×			
Liver	×	×	×	×	×	×	×
Lung	×	×	×	×	×	×	×
Spleen	×	×	×	×	×	×	×
Kidney	×	×	×	×	×	×	×
Brain	×	×	×	×			
Suprarenals	×	×	×	×			
Thyroid	×	×	×	×			
Thymus	–	×	×	×			
Lymphatic glands ...	×	×	×	×			
Haemolymph glands	–	×	–	–			
Pituitaries	–	×	×	×			
Bone marrow ...	–	×	–	–			
Salivary glands ...	–	–	–	×			
Skin	×	–	–	–			

The method of extraction adopted was that recommended by Kanitz, and is very simple. Glands were minced down, and pure glycerin added in the proportion of one-third gland and two-thirds glycerin. After two days' contact, the extract was strained through gauze, as lipases are easily injured by filtration.

The best medium known for extracting lipase from tissues is glycerin. Lipase is soluble in pure glycerin, as also are salts. Fatty acids, alcohols and esters are only very slightly soluble, but enough to aid lipolysis and also titration of these usually difficultly soluble substances. Glycerin also preserves the lipase by abstracting water, which injures the ferment. Glycerin is indeed well known as preservative, and anti-putrefactive, being used in commerce, for example in rennet preparations, etc., for that purpose. Hawthorn thought glycerin bactericidal to tubercle, but Fontes could not confirm this. Bacilli, seven days in 50 % glycerin at 38·5° C., did not lose in vitality or pathogenicity, also sputum kept by him for a year in glycerin, did not putrefy, but preserved the form and staining properties of the bacilli perfectly. It is doubtful how far the bactericidal influence which has

been ascribed to glycerin depends upon the glycerin itself. Glycerin is very liable to decomposition during purification by distillation, and may contain acrylaldehyde, and acrylic acid, which are bactericidal. This can be guarded against by testing for acidity. Again, if glycerin is boiled some decomposition may take place with the formation of these bactericidal products. At the beginning of this research all glycerin was boiled before use, with the intention of destroying any glycerin tolerant organisms which might be present, with the result that often embarrassingly bactericidal properties were developed. It was later heated not above 100° C. with satisfactory results. Glycerin is hygroscopic, absorbing water up to half its bulk, and for this reason should not be too concentrated when used with organisms. In the following experiments the glycerin present in the bactericidal mixtures was always less than 25 %, it having been previously ascertained that 50 % of glycerin was not bactericidal. Although ferment solutions extracted from organs are usually difficult to preserve from putrefaction, the glycerin extracts made from organs in this investigation all became sterile within a few days. That this did not depend on the direct action of the glycerin on the organisms, but rather on its preservative influence on the bacteriolytic substances present, was proved by the varying rate at which the organs became sterile. Liver became sterile at once; lung was perhaps the slowest of all. This sterility allowed of certainty in ascribing lipolytic action to the organ from which the extract was made.

Seven strains of human tubercle bacilli were used, *i.e.* H1, 20, 28, 67, 70, 79 and Arloing-Courmont strain, also four strains of bovine tubercle bacilli, B1, 4, 43, and 164, and five other acid-fast bacilli (Mist, Timothy Grass, Korn, Rabinowitsch, and Smegma). An emulsion was made in half-strength physiological saline solution. To do this, it was necessary in the case of the human strains, except Arloing-Courmont, to shake the bacilli in a shaking machine for two or three hours; the bacilli if shaken overnight may all be killed, so it is advisable not to overshake. The bovine strains were also occasionally shaken, though this was less necessary, as bovine tubercle bacilli usually emulsify better.

Bacteriolysis was tested in the following way: in each of a series of small sterile tubes were placed 0·25 c.c. of a different gland extract (glycerin $\frac{2}{3}$), then 0·5 c.c. half-strength physiological saline solution, then 0·25 c.c. bacillary emulsion. A part of the saline solution was sometimes replaced by sufficient $n/10$ NaOH to neutralise the known

acidity of the gland extract, or by weak $CaCl_2$ solution, or by bile. The gland extracts were always slightly acid, but not sufficiently so to affect the bacilli (Porter, *This Journal*, XVI. p. 66). The acidity of the bacteriolytic mixtures increased during the $2\frac{1}{2}$ and 24 hours of contact, at 37° C., or room temperature, but usually the resulting acidity was scarcely sufficient to account for the degree of bacteriolysis; however a part of the acid produced may have been absorbed by the bacilli, that is to say retained by the bacilli and not titrated. The addition of lecithin increased the bacteriolysis, but the acid split from it was sufficient to account for this. Esters scraped from egg medium together with the bacilli (and especially human strains are somewhat apt to eat into the medium and soften it) will, through splitting, increase the acid present. Bacteriolysis was apparently unaffected by the presence of weak $CaCl_2$, or of bile. When the bacteriolytic mixtures had been in contact, usually for 24 hours at 37° C., four drops were taken from each with a sterile pipette and inoculated on some medium. The Arloing-Courmont strain, and the acid-fast bacilli, other than tubercle, were inoculated on 3 % glycerin agar. As the other strains would not grow well on glycerin agar, Dorset was first used. This had to be discarded, as egg medium contains very many esters, lecithin, other glycerides, and cholesterin esters, so that strong lipase mixtures, notably those containing pancreas extract, ate into and liquefied the medium. B43 refused to grow on anything but egg medium, but those of the other strains which refused glycerin agar, yet grew well on glycerin-cholesterin agar. The bacteriolytic influence of gland extracts, like the lipolytic, slowly deteriorated, so that it was much lower in a few weeks' time, and practically non-existent in 2–3 months. Thus old inactive extracts, containing the same acid and glycerin, were available to control their former activities when fresh. It must be admitted that tubercle bacilli, though far less sensitive to acid than other acid-fast bacilli, are yet more sensitive to lipolytic gland extracts, either because they contain more fat (tubercle 40–44 %, Mist 16 %), or because the fats are of a different nature. Dead bacilli from lipolytic mixtures, for the most part stained perfectly with Ziehl-Nielssen and Gram stains, though a certain percentage lost their acid-fast properties. Proteolytic ferments affect the staining properties of acid-fast bacilli much more profoundly than do lipolytic. Dead bacilli, staining perfectly, rather suggest the effect of acid alone.

The following table shewing the bacteriolytic activity of the gland extracts investigated, also contains, for comparison, an indication of

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Gland extract	Esterases present in gland extract: Glycerin esterases	Lecithase	Higher esterases	Bacteriolytic power of gland extract on tubercle bacilli
Human Pancreas	+	+	weak	complete
Ox ,,	+	+	+	,,
Sheep ,,	+	+	+	,,
Pig ,,	+	+	+	,,
Human Liver	+	+	weak	,,
Ox ,,	+	+	+	,,
Sheep ,,	+	+	weak	partial
Pig ,,	+	+	+	complete
Cat ,,	+	+	+	,,
Rabbit ,,	+	+	+	,,
Guinea-pig ,,	+	+	weak	partial
Ox Thymus	+	+	+	complete
Sheep ,,	+	+	+	,,
Pig ,,	+	+	+	,,
Human Lymph Glands	weak	weak	weak	,,
Ox ,, ,,	+	+	+	,,
Sheep ,, ,,	+	+	+	,,
Pig ,, ,,	weak	weak	weak	,,
Human Suprarenals	0	,,	,,	partial
Ox ,,	+	,,	,,	complete
Sheep ,,	weak	,,	,,	partial
Pig ,,	,,	,,	,,	,,
Human Spleen	0	+	0	0
Ox ,,	weak	+	0	0
Sheep ,,	0	+	0	0
Pig ,,	+	+	0	partial
Cat ,,	weak	+	0	,,
Rabbit ,,	,,	+	0	0
Guinea-pig ,,	,,	+	0	0
Human Kidney (fatty)	+	weak	weak	partial
Ox ,, ,,	0	,,	0	0
Sheep ,, ,,	0	,,	0	0
Cat ,, ,,	+	,,	weak	partial
Rabbit ,, ,,	weak	,,	0	0
Guinea-pig ,, ,,	0	,,	0	0
Human Brain	weak	+	0	partial
Ox ,,	0	+	0	,,
Sheep ,,	0	+	0	0
Pig ,,	0	+	0	0
Human Thyroid	0	weak	0	0
Ox ,,	+	+	+	complete
Sheep ,,	+	+	weak	0
Pig ,,	0	weak	0	0
Human Lung	0	trace	0	0
Ox ,,	0	weak	0	0
Sheep ,,	weak	,,	0	0
Pig ,,	,,	,,	0	0
Cat ,,	+	,,	0	partial
Rabbit ,,	0	trace	0	0
Guinea-pig ,,	0	,,	0	0
Human Skin	+	weak	+	complete
Ox Bile	+	0	0	0
Pig Salivary Glands	0	weak	0	0
Ox Bone Marrow	+	,,	weak	partial
Ox Pituitaries	weak	,,	,,	,,
Sheep ,,	0	,,	0	0

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the esterases present in the same extracts. This table gives a brief summary of another paper wherein the distribution of esterases in the different organs is discussed in fuller detail (*vide* Porter, *Biochemical Journal*, 1916, x. p. 523).

Summary.

The results obtained point to a consistent relationship between lipolytic activity and bacteriolytic power on tubercle bacilli.

The least bactericidal extract was lung extract; the most powerful was pancreas extract.

Liver, thymus and lymphatic glands were strongly bactericidal.

Other organs, suprarenal glands, pig and cat spleen, human and cat kidney, human and ox brain, ox thyroid, cat lung, ox bone marrow and ox pituitary glands were found to be bactericidal to a lesser degree.

The human skin extract examined for bactericidal properties was fatty and cloudy in appearance and exceptionally rich in esterases[1]. Even if exceptional in its esterase activity this sample of skin bears out the relationship between lipolysis and bacteriolysis of tubercle bacilli in a striking way, as it was also extremely bactericidal.

No difference was noticed between bovine and human tubercle bacilli in susceptibility to any gland extract examined.

Other acid-fast bacilli, though on the whole less susceptible than tubercle bacilli to the influence of these extracts, were bacteriolysed by them. They were also killed by one lung extract (pig's) which contained an unusually large amount of olein lipase and which had no effect on tubercle bacilli.

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[1] Two other skin extracts were not cloudy and were comparatively poor in esterases, possibly due to the condition of the sweat glands at death.

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