SOME ASPECTS OF MENINGOCOCCAL VIRULENCE

A REPORT TO THE MEDICAL RESEARCH COUNCIL ON WORK CARRIED
OUT AT THE UNIVERSITY OF CAMBRIDGE FIELD LABORATORIES

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CONTENTS.

I. INTRODUCTION.

"VIRULENCE" is an ill-defined term and its use frequently gives rise to dis-
putes, which remain unresolved because the disputants have no common
basis other than that the term applies to organisms which cause disease. Thus
it is imperative that the writer clearly defines the meaning of the term virulence
as used in this paper, that the issue may not be confused by the reader
approaching the subject from a different point of view.

Since the term "pathogenicity" expresses the power possessed of microbes
to cause disease and since pathogenic organisms exist which cannot of them-
selves actively invade the living tissues of another organism, virulence is not
invariably a factor in pathogenicity; for the first limitation the writer places
on the meaning of the term virulence is that it shall only apply to organisms
capable themselves of actively invading the *living* tissues of the host. Not
only is this capability to invade actively and to multiply in the living tissues
of another organism very unevenly distributed amongst a relatively small
number of bacteria, but its expression, as determined by the aggressive and

defensive machinations of the organisms concerned, is so varied that almost every case has to be studied as a particular example of a multiplicity of factors which are exceedingly difficult to analyse. These factors or "forces" to a large extent form the object of study in infection and immunity, and our present knowledge does not allow of a complete appreciation of their existence and significance.

From the writer's point of view, *Virulence is the resultant of the opposed systems of forces, exerted physiologically by both the parasite and its host, in the efforts of each to maintain its life and health.*

The intensity of the aggressive mechanisms of the parasite and the intensity of the defensive mechanisms of the host vary independently, but the algebraical sum of these two quantities, which must of necessity have opposite signs, expresses the success or failure of the attempted infection; that is to say, the parasite either survives or is destroyed—it is virulent or non-virulent. Generally speaking, it is not essential for the host to die; the success of the parasite is sufficiently complete if it can maintain its life so easily as to cause the disease to-run its recognised clinical course.

But the need of the laboratory is not satisfied by this clinical manifestation, which could only be applied experimentally to animals subject to the disease it is desired to study, and then is limited by the powers of clinical observation, which are insufficient even in man.

Lacking a precise appreciation of the contributing factors, the measurement of virulence by a laboratory test must of necessity be a death struggle and its interpretation is not directly concerned with the mechanism whereby death is brought about; except in this far that it is essential for the investigator to be satisfied that the defeat of either protagonist is directly due to the other.

The virulence of a parasite is expressed, therefore, in terms of the death of one or other of the specified antagonists and a not unnatural bias has determined the indication of the result of this conflict to be Positive or Negative, according as the parasite or the host respectively conquers. For the same reason any process proper to the parasite, whether aggressive or defensive, is termed in this paper an "assailing factor" which means to say that it contributes to positive virulence.

The degree of virulence of bacteria is generally expressed in terms of the smallest dose administered in a living condition by a stated route, which constantly proves fatal to a chosen kind of experimental animal. Also it is customary to state that a given culture is non-virulent, or has lost its virulence for a particular experimental animal, when the experiment has resulted in the death of the parasite or, at least in the survival of the experimental animal.

Thus the purely relative significance of the term virulence requires a precise statement of the conditions of experiment, and for the purpose of this paper, the measure of virulence of the meningococcus is chosen to be the least amount of viable young culture, expressed in grammes, which, when injected

intraperitoneally at one time will cause the death of 20 gms. of mouse within 48 hours. When, in this paper, a culture is described as possessing "high virulence" or "low virulence," it is meant that the balance of interacting factors contributed by both host and parasite has favoured the parasite in the former case and the host in the latter.

II. Conditions Influencing the Determination of the Minimal Lethal Dose.

The estimation of virulence in its simplest form, resolves itself into the problem of determining the Minimal Lethal Dose of the living bacterium for a specified animal. Whether this is easy or difficult, is determined by two general conditions which themselves are very complex:

1. Whether or not the bacterium under investigation causes natural disease in the available experimental animal.

2. The nature, degree and rapidity of alteration taking place in the bacterial culture under the conditions imposed by growth on artificial nutrient media.

Now, in relation to the first condition, there is no known experimental animal which naturally affords the conditions required by the meningococcus to reproduce the characteristic human disease. It is true that von Lingelsheim (1905), Flexner (1907) produced meningitis in monkeys, by the extreme procedure of sub-dural inoculation with meningococcal cultures and that Weinberg (1909) described a case of spontaneous meningitis in a chimpanzee due to an organism very closely resembling the meningococcus. Also, that a meningitis of horses, goats and sheep is known, which is caused by the *Diplococcus intracellularis equi* (Johne, 1896) of which the morphology and cultural characters differ very little from Weichselbaum's coccus and a clinical, epidemiological and pathological account of the disease of Borna would amply describe cerebro-spinal fever in man (Cadéac, 1914). Nevertheless, it is possible to inject large quantities of virulent meningococcus culture, freshly isolated from human cases, intravenously into horses without causing meningitis and Robertson-Milne (1906) failed to produce meningitis in goats by sub-dural inoculation with meningococcus culture.

Thus, although the usual experimental animals can be killed by dosing them with meningococcus or its products, it cannot be claimed that they are susceptible to that parasite in the same way that man is. Difficulties are to be anticipated therefore and relatively large doses may be expected to be necessary to establish the desired minimal lethal dose.

It is important to notice in this connection, considering the fact that the typical disease is a meningeal infection and the relatively large number of cases which recover without specific treatment, that the meningococcus must possess a relatively low grade of virulence.

A mere glance at the literature of meningococcal infections imposes an embarrassment of choice between the numerous and varied culture media ardently recommended, and almost every serious entrant into this field of

research introduces either a new medium or some modification of an old one. So marked is this feature that there is ample justification to anticipate difficulties arising in relation to the second condition expressed above; nor need there be any hesitation in attempting to add another item to the menu, since it is evident that the ideal medium has not yet been found.

For these reasons it comes about that the feeble and irregular killing power of the meningococcus is emphasised throughout the literature, and Dopter (1921, p. 425) summarises the situation, after describing how the virulence of a culture varies from day to day, by saying: "The minimal lethal dose is impossible to define." Horder and Gordon (1907), Gordon (1917) expressed the resistance of experimental animals in terms of the number of doses they could support administered at intervals of one hour, though in later work Gordon (1920) used single doses, but not without disappointment.

The writer claims to have probed some of the more important conditions it is essential to recognise and by so doing to have made it possible to establish a minimal lethal dose for the meningococcus. These conditions will now be briefly outlined.

(a) *Viability and purity of culture* are obviously essential conditions, but the necessity for control cultural and microscopical examinations requires emphasis, on account of the peculiarly sudden way in which a meningococcus may die under apparently favourable conditions. Failure of an experiment is occasionally adequately explained by reference to these controls. At the same time there is no evidence that the meningococci capable of surviving in artificial cultivation represent the sum of the viable cocci *in vivo*, therefore a colony count is not considered necessary, but it is required to prove that a sufficiently large proportion of cocci are still living.

(b) *The health of the experimental animal* is of primary importance as survival or death is the indicator used to read the result of the experiment. It is not possible to give too much attention to the breeding and care of experimental animals. Post mortem examination frequently reveals the cause of an anomalous result to be intercurrent disease and this applies particularly to mice which have been bought from a dealer.

(c) *The general influence of the nutrient medium* used is of considerable importance, but its exact degree is undetermined. The growth requirements of the meningococcus have been given a great deal of attention and this subject must be dealt with in a separate report, although certain features will be considered briefly here insomuch as they bear directly on the subject. A variety of media have been used, such as Gordon's trypagar, Nicolle's M.M., peptone serum agar, etc., and all present intrinsic difficulties to attempted rigorous standardisation, therefore other methods have been devised to meet certain difficulties which will be described elsewhere. Fluid media have been avoided on account of the difficulty they present to accurate measurement of the quantity of growth used as a dose and the poor yield compared to growth on agar media.

The value of an agar medium for growing the organism is estimated by the average weight of dried growth obtained from unit area; this has been used as an index of uniformity in various batches, and a very large number of such comparisons have been made. With any medium there is an appreciable variation in the growth obtained in different subcultures of a given strain on the same batch of medium and this depends partly upon the surface moisture of the medium, which can be controlled to some extent by observing the precaution of having the medium and plates at the same temperature at the time of pouring, partly upon the depth of the medium, but more particularly upon the phase of growth of the organism which will be mentioned presently.

Not only do the amount and physical properties of the growth vary with the various kinds of medium, but there is complete disagreement between the yields of different batches of a given medium, made as ordinarily described; the extreme variation for a series of batches of trypagar is as much as 25 per cent. on either side of the arithmetical mean yield of cocci per unit area of that medium. For this reason any technique by which animals are dosed in terms of agar-slopes, Roux-bottles, etc., alone will be beset by an enormous error which is still further multiplied when the variation in percentage of moisture is taken into account; it is calculated that the total error due to variation in growth by such a method of measuring doses of living meningococcus may easily amount to 100 per cent., which makes it quite impossible to compare any two experiments. It must be stated that very considerable variations in yield per unit area are not appreciated by simple inspection.

Even when the dose of living meningococcus is accurately weighed, thus eliminating the error due to variation in mass of growth per unit area, a considerable error is introduced by the variation in the percentage of dry coccus represented in the growth obtained on the ordinarily described media; the figures for a series of batches of trypagar show a variation of 20 per cent. on one side and 13 per cent. on the other side of the arithmetical mean of the determined percentage content of dry cocci in the moist growth allowing of a possible error of 33 per cent. in dosing animals with living cocci.

As the variation in percentage of moisture on each side of the mean for a given batch of such a medium, is much less than the variation on either side of the mean for a number of different batches, it is evident that the method of making the medium does not supply a standard material.

The complicated ingredients required for bacteriological media to grow obligatory parasites, present considerable difficulties to the consistent realisation of the optimal conditions necessary to obtain uniformity of growth. But, so far as the meningococcus is concerned, the medium we have attempted to standardise has reduced the range of variation to a considerable extent; for, not only does the determined percentage of moisture exhibit the same arithmetical mean for a given batch as for a series of batches, but the figures show a variation of − 5 per cent. on one side and + 7 per cent. on the other side of the average determined percentage of dry cocci represented in the moist

growth, thus allowing a possible error, due to variation in moisture, of 12 per cent. in dosing animals with living cocci; and this error is further reduced to 7 per cent. by basing all calculations on the mean percentage of dry cocci. These results seem to indicate that further improvement is possible and it is evident that, in determining the minimal lethal dose of the living meningococcus, it is of primary importance to estimate the errors which the nutrient medium may introduce.

(d) *The correct measurement of the dose of living cocci* is a matter of difficulty, because the problem of estimating the proportion of viable cocci in a mass of growth has not been solved. It has been found by repeated sub-culture of the meningococcus at a 24 hour interval, that, on a medium giving the most luxuriant cultures, there is a wave of growth, indicated by a progressive increase in yield per unit area of medium followed by a sudden drop to minimal growth. The range of this fluctuation is usually in the region of + 20 per cent. and − 12 per cent. on the arithmetical mean yield for a good medium and is not appreciable to the eye, but on certain media the drop in growth can readily be perceived by inspection. This wave of growth appears to have a fairly regular period and in our experience the drop in the curve occurs every third day during the early part of a long series, but the interval becomes shorter as the sub-cultures are continued. Apart from any suggestion as to its immediate cause, this periodic rise and fall in the number of viable cocci contained in a given mass, is of profound importance with regard to the accurate measurement of dose in experiments depending upon the viability of the cocci. A virulent strain tested in the transition generation, might very easily give results showing an apparent loss of virulence, whereas in all probability a large proportion of the cocci injected were dead.

In dealing with very large masses of cocci, as is frequently necessary and desirable, it becomes important to take into account the specific volume of meningococcal growth; it has been determined by experiment that if this factor is taken as 1·0, it is well within the range of other experimental errors.

Still greater accuracy obtains if all doses are correlated in terms of the percentage of dry bacterial protoplasm, determined on an aliquot part of the actual growth used for any experiment.

(e) *The strict correlation of dose to body weight of animal* very materially influences the regularity of the result obtained: the inoculation of an animal with a known weight of culture amounts to diluting the virus with mouse, rat, etc., and it is quite as important to measure the amount of animal used as it is to know the amount of solvent required to dilute a standard solution to a desired degree.

The following method adopted by the writer facilitates this undertaking and depends upon the fact that if an emulsion is made so that the dose for 20 gms. of mouse is contained in 1 c.c., then the dose for a mouse of any weight will be as many 1/20th parts of a c.c. as the number of grammes the mouse weighs.

The dose is given in proportion to an arbitrarily chosen unit of animal (20 gms. of mouse, 100 gms. of rat, etc.) and, for this purpose, every animal is weighed to the nearest twentieth of a unit, which factor is determined by the error the physiological processes of the animal might introduce into the weighing and is proportional to the general error of experiment. These weighings take very little time and very soon become established as a routine procedure. The required dose for an animal of any weight is obtained thus: let the chosen unit of animal be denoted by U; now dilute the substance it is desired to inject so that the dose for xU gms. of animal is contained in a volume V c.c., where x is any whole number. The volume of the dose of this dilution required for any animal less than xU gms. weight is given by the formula:

$$\text{Dose} = \frac{\text{Weight of Animal} \times d}{x \cdot U} \cdot V \text{ c.c.} \quad \dots\dots\dots\dots\dots(1)$$

or more conveniently:

$$\text{Dose} = \left(\frac{\text{Weight of Animal}}{F}\right) \cdot g \cdot V \text{ c.c.} \quad \dots\dots\dots\dots\dots(2)$$

where g is the smallest convenient fraction of the volume V which can be injected into the animal accurately (*e.g.* $V/20$) and

$$F = \frac{U \cdot g}{d} \cdot x \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots(3),$$

and d is the fraction of the full dose it is required to give per unit of animal (*e.g.* 1, 1/2, 0·75, etc.).

If, in determining the volume of the dose for a given animal, V is taken as unity and g as fractions of V in divisions of the graduated instrument used for measuring the dose, then if the part in brackets of equation (2) is worked out it gives the number of divisions of the value of g which have to be injected. This part of the calculation can usually be done mentally and by this means it is as easy and as rapid a process to inoculate a number of animals of varying weights as if all were of the same weight.

It follows from equation (3) that the value of F varies inversely as the value of d, and in practice it is found that F determines the maximum and minimum weight of animal to which it is reasonably possible to give a desired dose; therefore, the smaller the value of d the greater the weight of the largest animal which can be used conveniently, when x represents a determined integer.

This method is capable of general and easy application and consequently animals can be dosed in strict proportion to their weight, whatever it may be, without the tiresome necessity of selecting a sufficient number of animals of specified weights for a particular experiment; an ideal seldom realised.

(*f*) *The influence of sub-culture and age* are extremely important in their capacity for decreasing virulence and the writer knows no medium which will fail to exhibit the well-known attenuating influence on the meningococcus of repeated sub-culture, even when practised at fairly short intervals (12 hourly). Therefore it is very important, when comparing the virulence of cultures of

the meningococcus, only to use growths which have been subjected to the same number of generations on a standard routine medium; the question dealt with in subsection (*d*) seems to require that the second generation from the stock culture should be used.

In the course of this investigation the stock cultures have been maintained on Dorset's egg medium in paraffin sealed tubes, but experiments are in progress with other media. The writer has taken the precaution of keeping a record of every sub-culture made of any strain in the form of a genealogical tree and the growth used in each experiment is carefully correlated with this record; thereby certain features of the ageing of cultures have been noticed, of which the following are examples.

Starting with a culture on egg of known virulence and inoculating mice only with the second generation on strictly comparable media, the observed decrease in virulence with age is shown in Table 1. The transitory recovery of the virulence with sub-culture *on to egg medium* is to be noticed, but the duration of this degree of virulence is less than in the case of the preceding culture and repetition of the process is without effect, perhaps because the influence of repeated sub-culture intervenes.

Table 1.

	Age of culture in days			
Egg A	Egg B from egg A at 347 days old after raising virulence	Egg C from egg B when 51 days old	Egg D from egg C when 17 days old	M.L.D. for 20 gms. of mouse, in grammes of living meningococcus
1	—	—	—	0·0080
16	—	—	—	0·0080
70	—	—	—	0·0120 (=50 % M.L.D.)
347	—	—	—	0·0160
Sub-cultured 5 generations on egg and 96 on agar	—	—	—	Virulence raised by *in vitro* method and sub - cultured on to egg B
—	5	—	—	0·0040
—	12	—	—	0·0040
—	26	—	—	0·0040 (=50 % M.L.D.)
—	27	—	—	0·0080
—	40	—	—	0·0160
—	51	—	—	Sub - cultured on to egg C
—	—	5	—	0·0040
—	—	12	—	0·0160
—	—	17	—	Sub - cultured on to egg D
—	—	—	1	0·0160

M.L.D. =Minimal Lethal Dose.

The accumulation of such observations has enabled the writer to gauge the virulence of a culture or to select a culture of required virulence for some particular experiment by reference to the "genealogical tree," which has justified its existence by economy in the lives of animals and in augmenting the precision of experiments.

(*g*) *Summary.* In order to establish the minimal lethal dose of living meningococcus culture, that is, to ensure that it will not vary from day to day in an arbitrary manner, it is important to observe:

(1) That a sufficiently large proportion of the cocci used are viable.

(2) That the experimental animals are in good health.

(3) That the nutrient medium affords a growth of approximately constant properties (particularly percentage of moisture).

(4) That the phase of growth is on the up grade.

(5) That the dose bears a strict relation to the body weight of the experimental animal.

(6) That the culture is not unduly aged nor has been repeatedly subcultured since the last time of testing its virulence.

III. The Graduation of Doses and the Selection of the Minimal Lethal Dose.

The graduation of a suitable series of doses of living bacteria and the selection of that amount which can be designated one minimal lethal dose involves problems which are, at the same time, of paramount importance and unsolved. Consequently the suggestions presently to be made are to be considered as opinions, based upon experience, which serve very well as a guide until they can be replaced by a rule deduced from proved facts and capable of general application.

The whole question involves the complicated balance maintained by influences contributed by both parasite and host and therefore it is insufficient to base a system of doses upon properties of the parasite alone. It may be considered that the various influences involved resolve themselves into two complex factors which may be conveniently expressed as the "capacity factor" and the "intensity factor."

For want of more precise methods both of these factors are considered by the writer in terms of mass of parasite; a procedure which is inadequate, particularly so in the case of the capacity factor. The capacity factor is regarded as the mass of parasite the actual presence of which is incompatible with the survival of the host and its precise measurement has not been achieved; nor is it possible at the present time to anticipate its full significance. Notwithstanding this difficulty, it *is* possible to estimate whether the capacity factor diverges widely from, or approximates to the measured dose received by a given animal, and it is this "index of adaptation" which expresses the degree to which a parasite is adapted to invade a given experimental animal. Where the capacity factor approximates to the measured initial dose, as in the case of the meningococcus, the graduated doses used to titrate the minimal lethal dose of a culture, need to vary by relatively large amounts in order to obtain clear cut results; for it must be remembered that under these circumstances the actual amounts injected must be large as it is almost a question of overwhelming the animal by the dose itself. This condition contrasts

strongly with the case of parasites better adapted to invade laboratory animals, where the capacity factor diverges widely from the initial measured dose (*e.g.* pneumococcus), where the reverse is true because the animal host is overwhelmed by the ready multiplication of organisms introduced in small numbers. It appears that the capacity factor is a variable dependent on the systems of forces contributed by both parasite and host which determine virulence, and there is, at present, no indication of the possible range of variation of this factor. But it seems to the writer that another factor is needed to account for the apparently irregular variation of the estimated index of adaptation and such that it may be considered to determine the magnitude of the initial measured dose required to allow of the capacity factor functioning. This second factor, the intensity factor, is associated with the fact that the significant difference between effective doses diminishes as the quantity constituting a minimal lethal dose decreases. This requires that the difference between the small doses in a series be much less than the difference between the large doses. Therefore a series in arithmetical progression is theoretically unsuitable and one in geometrical progression satisfies the requirements.

For the present the conditions for the meningococcus are amply satisfied by a series in geometrical progression, because of the large doses required, the magnitude of the amount constituting a significant difference, and the variations introduced by the imperfectly adapted circumstances of cultivation of that organism *in vitro*.

The value of the common ratio of a series is determined by both factors: the index of adaptation, which depends upon the capacity factor, is a guide which suggests whether the successive doses are to vary by large or small amounts, and the intensity factor, gauged by the magnitude which constitutes a significant difference in the region of one minimal lethal dose, suggests how large the common ratio need be. The amount which constitutes a significant difference is best estimated by comparing the doses which are just sufficient to kill certainly with those which will kill some though not all of the animals so dosed.

It may be expected, from what has been said in this and the preceding section, that the meningococcus requires a large initial dose which approximates to the capacity factor, therefore the common ratio for a series of doses of that organism in geometrical progression will need to be of a low value. In the writer's opinion 0·5 minimal lethal dose of meningococcus is to be considered small in comparison with 1·0 minimal lethal dose, because such a dose kills comparatively few animals; that is to say, that $(1/2)$ is reckoned small in comparison with unity; $(1/2)^2$ is a magnitude of the second order, etc. Therefore, for a series of doses of living meningococcus in geometrical progression the common ratio adopted is 2, when mice are used. For the sake of comparison, it may be stated that 10 has been found to be a suitable common ratio for a similar series of doses of pneumococcus for mice.

The selection of that amount which constitutes one minimal lethal dose

is always a matter of difficulty, because of the variation in resistance of individual animals and the margin on either side of any selected dose within which a slight variation might give a different result to that obtained.

So far as the animal is concerned, the essential condition which must be imposed is that the dose, when the total mass is given at one time by a stated route, shall be sufficient to kill all the animals so dosed; it must be what Pasteur called a 100 per cent. fatal dose. From this it follows that owing to individual variation in resistance only certain of the animals will receive exactly one minimal lethal dose and the rest rather more than sufficient to kill. The other difficulty is not so easily overcome because no very definite limiting requirement can be set to control it. The starting point of a series of doses is determined by such factors as measurable quantities, volumes which are safely tolerated by the animal of choice, the number of available animals, etc., but more particularly by the judgment of the investigator born of experience. It is important only to maintain a technique which enables the various experiments to be faithfully comparable amongst themselves and readily repeated by other observers.

Thus it is that what is designated "one minimal lethal dose" of a parasite, cannot be considered to represent the point on a curve where the defence of the host reaches zero, but, rather, a selected field in an area representing that phase of a system in which all the hosts die.

IV. The Variation in Virulence of Freshly Isolated Strains.

During the course of these investigations opportunity for examining freshly isolated strains has been insufficient to allow of definite conclusions.

The writer is indebted to Dr M. H. Gordon, C.B.E., C.M.G.; Dr A. Stanley Griffith, Major A. S. G. Bell, O.B.E.; and Dr W. H. Scott for the strains he has received. Almost all the cultures available are very old, in some cases dating to 1915, and repeated sub-culture has deprived them completely of whatever virulence they may have possessed.

The scarcity of material only allows of the statement that the freshly isolated strains varied considerably in virulence, as measured by experiments with mice, and that this property exhibited some degree of independence of the endotoxin value of the strain. Gordon's experiments (1920, p. 23) show instances of similar conditions and it is hoped that it will be possible to investigate these points when the arrangements for the collection of freshly isolated strains, which are being made on the part of the Medical Research Council, have been completed.

The need for such an investigation is emphasised by Gordon's (1920, p. 22) observation "That the pathogenicity of the coccus alive and dead, *i.e.* between the virulence and toxicity, was far less in the case of the older cocci than in some, though not in all, of the most recently isolated ones."

V. THE FATE OF MENINGOCOCCI INJECTED INTRAPERITONEALLY INTO MICE.

The establishment of the conditions required for the determination of a minimal lethal dose for the living meningococcus, of itself a matter of interest to immunologists, is of importance because it allows of the investigation of questions relating to the virulence of that organism and the failure to define this primary unit has resulted in the impossibility of titrating the anti-infective power of anti-meningococcal serum.

The limitations of our knowledge of the virulence of the meningococcus is summarised by Dopter (1921, pp. 425–426) and emphasised by the failure experienced by such experimenters as Dopter, Kolle, Wasserman, Gordon, Amoss and others in all attempts to raise the virulence of that organism.

In order to appreciate the conditions determining the raising and lowering of the virulence of the meningococcus to be examined in this paper, it is essential to study the fate of the cocci injected intraperitoneally into mice and rats.

During the early stages of the investigations it was desired to select the more virulent of the available stock cultures for use as antigens in immunising horses. For this purpose mice were used as the test animal and the most favourable route was found to be intraperitoneal. However, it proved very difficult to kill mice with any degree of certainty by injecting living meningococci of strains which had been kept so long in culture.

When an occasional mouse, perhaps less resistant than its fellows, succumbed, the meningococcus was sought for in its body fluid by microscopical and cultural methods, usually without success. But as very large doses of cocci had been introduced into the peritoneal cavity it was inconceivable that all trace of these should be completely obliterated during the time the mouse survived, not more than 14 to 20 hours. In the post mortem examination of such mice, besides the general signs of a purulent peritonitis, chiefly indicated by the character of the scanty peritoneal exudate in which meningococci could seldom be found, the most striking feature of the abdominal cavity was the tightly rolled up omentum. It was here, embedded in the omentum and within its folds, together with masses of phagocytes, that the meningococci were found in large numbers and the picture presented by smears of this organ were suggestive of the fight in progress.

Durham (1897) first observed this collecting action of the omentum, in connection with staphylococci, and Roussiel (1911) has shown that the concentration of phagocytes is always much greater in the omentum than in the peritoneal cavity.

But the uncertain effect of these ancient strains complicated the investigation and it was not until freshly isolated strains were available that the fate of the meningococcus, when injected intraperitoneally, could be examined more extensively, for then it became possible to kill experimental animals more frequently and to compare the effects of variation in virulence.

A mouse dying as the result of multiple intraperitoneal minimal lethal doses of a virulent strain, may exhibit in the peritoneal exudate very large numbers of healthy-looking, well-stained meningococci, of which comparatively few are found to be intracellular. That the presence of such large numbers of cocci in the peritoneal fluid partly depends upon the animal having received an overwhelming dose, is suggested by the examination of other mice, which have died in the course of the same experiment when it is found that the number of cocci seen noticably decrease with the fall in the dose. It is usual, when working with doses of the magnitude of only one or two minimal lethal doses of a strain of moderately low virulence, to experience difficulty in discovering many meningococci in the peritoneal exudate and quite frequently none can be found. When present they are usually both intra- and extracellular, and it cannot always be said in which situation they predominate. The influence of increased virulence is to augment the number of cocci to be found in the peritoneal exudate and to leave no doubt that the majority are extracellular; but the above-mentioned relation to mass of dose is still apparent. It is under these circumstances that cultures can be obtained from the peritoneal exudate and from the heart blood.

Turning now to the omentum, it is a striking fact that when living meningococci have been injected intraperitoneally, it is only on very rare occasions that cocci cannot be found in this situation; even when they have not been discovered in the peritoneal exudate they have been abundantly present in the omental smear. In the omental smear of a mouse dead as the result of an intraperitoneal dose of living organisms, the extracellular meningococci are in the majority and it is often surprising what massive numbers of cocci are to be seen.

A feature of importance to be considered here is the cytology of the inflammatory exudate. Very large numbers of cells are to be found in the folds of the omentum, but in the free peritoneal fluid they are usually less numerous and they may be scarce when the cocci have been swept up cleanly or when the animal's reaction is poor. The type of cell found under such circumstances as the present, and the time and sequence of their appearance have been thoroughly described by Bordet (1920, p. 173), Dopter (1921, pp. 85 and 99) and others and need not occupy us here, where the character of chief interest is the state of health of the cell.

When the total mass of meningococci, intra- and extracellular, in a given situation is small, the cells for the most part stain normally, but when very large numbers of cocci are present very few healthy looking cells of any type are to be seen; the majority, then, are in various stages of degeneration and fragmentation of the nuclei is a common feature. But this statement needs qualification, for although mass of cocci seems to be the preponderating factor, the observed phagolysis is really proportional to what might be expressed as the "mass of assailing factors."

Thus, remembering the relative distribution of the cocci in the peritoneal

fluid and the omentum, the picture which each situation presents can be realised in all its variations. In animals which have succumbed to a moderate dose, it is common to find that the peritoneal fluid is scanty, almost clear, the few cells seen are healthy, and that cocci are absent or difficult to find; while, on the other hand, the rolled up omentum encloses the exact opposite state of affairs, abundant cocci and very marked phagolysis.

With regard to the ingested cocci and those whose freedom has been regained by the rupture of a cell, various degrees of granular degeneration, typical of intracellular bacteriolysis by leucocytes, are indicated by their stained appearance; and it is a matter of more than passing interest that the amount of degeneration, exhibited by phagocyted cocci, appears to be to some extent in inverse proportion to the number of cocci present in the cell. Although many such altered cocci are to be found free in the inflammatory exudate bearing no obvious relation to a cell, it is not always possible to say whether their condition is due to intracellular lysins and subsequent liberation, or whether leukines liberated by disintegrated cells or bacteriolysins and alexine have brought about the change; autolysis is not a probable factor. An opinion can sometimes be based upon the grouping of the cocci and the amount of evident phagolysis but even when the influence of leucocytes is strongly suggested there is still ample scope for the action of bacteriolysins in the ordinary sense.

Such are the variations in the microscopical appearance of the inflammatory exudates of mice which are the victims of living meningococci and it is important to compare them with the conditions found in mice killed by injecting killed cocci and with mice that have successfully resisted a dose of living cocci. Thus, though derived from a highly virulent culture, when cocci killed by heating at 55° C., or by desiccation over sulphuric acid, neither of which operations seems to interfere with the endotoxin of the meningococcus, have been dosed in sufficient quantity to cause the death of the animal, it is very rarely that any trace of them can be found in the peritoneal fluid and when present they are almost invariably intracellular. Under these circumstances, too, the omentum is tightly rolled and it is there again that the cocci are found, but it is a striking feature that the majority are intracellular though there is marked phagolysis and liberation of partly lysed cocci. It has been the writer's experience in inoculating a large number of animals with meningococci and their products, that if a mouse or rat looks anything other than extremely ill about 24 hours after inoculation it is unlikely to die, and, if at that period it is sufficiently well to take the least interest in food or its personal comfort it will certainly recover.

Now, 24 hours after inoculation with living virulent cocci, if mice or rats, then so well as to feed and clean themselves, are killed and examined immediately, a condition is found which very vividly contrasts with what has been discussed above. There is an abundant turbid peritoneal exudate containing very large numbers of healthy cells and numerous macrophages busily

ingesting such damaged cells, with their contents, as may be present. The vast majority of cocci are intracellular and whether or not there are *any* free cocci, and also the degree of damage to cells, depends again upon the mass of the dose. Further, and more striking still, the omentum, only rolled at its margin if at all, is frequently stretched between the coils of intestine in all directions, as if when the fight is going well for his side the important stratagem is to increase the area of conflict and provide a large surface for the diapedesis of leucocytes and so to launch these to the attack actually where they are needed. An omental smear presents the same picture as the peritoneal fluid only the concentration is greater.

Three facts of immediate interest must be stated here, though they will receive full discussion in another paper where they properly belong.

(1) The endotoxin of meningococci is liberated, apparently unaltered and in its full strength, both by the leukines extracted from polymorphonuclear cells and by the sensitising substances and alexine of normal serums.

(2) Freshly isolated strains of meningococci have been examined which possess a high degree of virulence and a low endotoxin content. Living doses of the former kill readily and the latter with difficulty, while doses of whole dead cocci of either strain kill with great difficulty, in accordance with what has already been described.

(3) The minimal lethal dose of dead cocci required to kill 20 gms. of mouse represents very many times the minimal lethal dose of extracted endotoxin of the same strain.

It must occur from time to time, in experiments of the nature required by this type of investigation, that, though the animals receiving the larger doses of a series die regularly one or more of those receiving the smallest doses die, although similarly or even more highly dosed animals do not at any time show signs of inconvenience. Irregular results of this kind may be interpreted in the light of what has been said above. On examination the peritoneal fluid and omental smear may show that a good fight has been attempted and considerable phagocytosis accomplished, with the result that relatively few healthy looking free meningococci are found. Nevertheless, for some reason, there is extensive phagolysis and many partially lysed meningococci are seen in the region of degenerated cells. In such a case the interpretation may be allowed that the endotoxin liberated by the leukines has turned the scale. In other cases phagocytosis has been less complete and free cocci are abundantly present; this state of affairs may be labelled "susceptible animal" or "lowered resistance" without advancing matters and frequently a closer approach to the truth is difficult to attain, though sometimes a relatively trifling inter-current disease may be accepted as explaining the difficulty. An experimental reproduction of this condition, faulty phagocytosis, will be dealt with in the next section.

This state of affairs must not be confused with that which may be described as the survival of animals receiving "multiple minimal lethal doses," a diffi-

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culty very seldom experienced by the writer when using *living virulent* cocci, then only in an individual animal and so probably due to some error introduced accidently. Very irregular results may obtain when working with cultures of low virulence, which may be due either to predominance of non-resisting cocci, or as will be discussed elsewhere, may be due to the lack of some quality in the medium.

From the point of view of virulence the prominent features of the foregoing considerations may be summarised as follows:

(1) The preponderance of free meningococci in the inflammatory exudate is proportional to the virulence of the strain.

(2) When entire meningococci are ingested and *retained* by the phagocytes they cease to take any further part in the conflict and undergo degenerative changes.

(3) In the face of failure of phagocytosis, the power of the leukines liberated through phagolysis is relied upon to destroy the invading organisms, but this is not always successful; with virulent strains many cocci will be found which take the stain deeply and such are often grouped in small clusters as if multiplying rapidly.

(4) When the influence of the meningococcus is such that extensive phagolysis with consequent bacteriolysis is brought about, the endotoxin thus liberated favours the meningococcus in the conflict.

(5) When victory is in the balance the omentum strives to confine the area of conflict and diminish the surface for absorption of toxic products.

But when the experimental animal is definitely master of the situation, the omentum is frequently found spread out and the field of operations is thus extended to the full.

VI.   Interference with the Activity of the Leucocytes and the Peritoneal Folds.

The evidence cited in the preceding section suggests that, whatever the physiological forces at work may be, the virulence of the meningococcus can be expressed in terms of resistance to phagocytosis, and such a view would agree with statements which have been made in respect of other organisms (Bordet, 1920, p. 209; Levaditi, 1914, pp. 484–486). The present section will be devoted to the discussion of further experimental evidence bearing upon the above inference, but it must be recognised at the outset, that, were resistance to phagocytosis the only term of reference used to express virulence, an incomplete statement would result, even though phagocytosis may be the principal factor in natural immunity.

Although the mechanism immediately responsible for the death of the animal properly belongs to another investigation, it is of more than passing interest that individual animals are found to exhibit less than the average degree of resistance to meningococcal infection. Such a state represents an *apparent increase* in virulence of the culture used and the problem is thereby

posed as to the possibility of reproducing the condition in the experimental animals of average powers of resistance.

Besredka's important experiments (1899) demonstrate that the amount constituting a fatal intraperitoneal dose of As_2S_3 varies inversely as the number of leucocytes present in the peritoneal cavity, and, further, that animals which have previously received an intraperitoneal dose of finely powdered carmine succumb to a dose of As_2S_3 which normally they would have tolerated. The effect of the carmine is to encumber the leucocytes.

Roussiel (1911) describes similar experiments, particularly in relation to the function of the omentum and shows that when the pedicle of the spleen has been ligated death of the animal invariably supervenes only when the omentum has been put out of action by excising it, or by a previous dose of carmine, or carbon.

• In the literature concerned with the problem of phagocytosis there are numerous instances demonstrating that interference with the activities of the leucocytes favours infection by bacterial parasites but very generally the authors are content to use their experiments as arguments contributing to the controversial discussion of humoral and cellular immunity and the point of view of the parasite is usually neglected.

In the present section, the evidence of experiments interfering with the cellular defence of the host will be considered only in its relation to the raising and lowering of the virulence of the parasite.

An intraperitoneal dose of 0·01 gm. of carmine powder in no way affects the apparent health of mice and the subsequent condition found varies with the time which elapses between dosing and killing the animal for examination. Recoux (1897), Durham (1897), Heger (1904), and others show that carmine is taken up almost completely by the omentum within 15 minutes of intra-peritoneal injection. It is first "agglutinated" into masses by mucin as described by Gengou (1908) and then gathered up by the peritoneal folds; the omentum is the principal of these, but the pelvic folds and those in relation to the genital organs are almost equally active. At an early stage (12–20 hours) carmine has no other visible distribution but microscopical examination shows that a fair proportion of leucocytes wandering in the peritoneal fluid contain carmine. The peritoneal folds are tightly rolled and literally crammed with carmine and leucocytes. Four days after injection the carmine is more widely distributed; besides being massed in the peritoneal folds it chokes the lymphatics of the diaphragm and mesenteries, colours the lymphatic glands of the abdomen and thorax and is found in the liver, spleen, and even, contained in leucocytes, in the heart blood and lungs.

The leucocytes free in the peritoneal cavity contain carmine. After three weeks there is no free carmine in the serous cavity but the peritoneal folds are still tightly rolled and crammed with carmine and leucocytes, many of which are overburdened with the pigment. The larger masses of carmine are almost embedded in leucocytes. Carmine is very readily taken up by leucocytes but

to other substances such as finely divided chemically pure carbon and aleurone they appear to behave rather differently.

Numerous experiments with living meningococci, on the lines of those of Besredka with As_2S_3, show conclusively that in the presence of carmine, given either some hours before or at the same time as an injection of cocci, the apparent virulence of the culture is markedly raised. The following experiment with a strain of moderate virulence will serve as an example.

An 18 hour culture of Type I Netley, second generation from a 27 days old egg culture, was suspended in 0·85 per cent. solution of NaCl in a concentration of 0·0080 gm. living per c.c. The other substances injected into the mice were a 1 per cent. suspension of levigated carmine and 2 per cent. peptone broth, both sterilised in the autoclave for 20 minutes at 120° C. Each dose was duplicated for the result of inoculating single mice is often misleading. The result of these inoculations is shown in Table 2.

Table 2.

Intraperitoneal dose of

| | Carmine in gms. | | Broth in c.c. | Meningo. gms. | |
	At same time as meningo.	96 hrs. before meningo.	18 hrs. before meningo.	living per 20 gms. of mouse	Result
A	0	0	0	0·0080	Both died
	0	0	0	0·0040	One died
	0	0	0	0·0020	Both lived
B	0	0·005	0	0·0080	Both died
	0	0·005	0	0·0040	One died*
	0	0·005	0	0·0020	Both died
C	0	0	1·0	0·0080	Both died
	0	0	1·0	0·0040	Both lived
	0	0	1·0	0·0020	One died
D	0	0·005	1·0	0·0080	Both lived
	0	0·005	1·0	0·0040	One died
	0	0·005	1·0	0·0020	One died
E	0·005	0	0	0·0080	Both died
	0·005	0	0	0·0040	Both died
	0·005	0	0	0·0020	Both died
Control	0	0·005	0	0	Killed and examined at the time that the other mice were inoculated with meningo.

* The greater part of the dose of meningococcus leaked back after injection in the mouse which survived this dose.

Post mortem examinations. The control mice were killed and examined at the time the other mice were inoculated with meningococcus and showed the typical appearance of mice dosed with carmine four days previous to examination. It is only necessary to emphasise the fact that the carmine found in the peritoneal exudate was all intracellular and that many cells were crammed with pigment.

Series A. These mice revealed the picture that has already been described under such circumstances as the present, within the usual limits of variation. It suffices to emphasise that masses of cocci were present in the tightly rolled omentum and that phagolysis and bacteriolysis were marked.

Series B. Here the distribution of carmine only differed in degree in different situations according to the animal and the general picture of the carmine was identical with that exhibited by the control mice. There were, however, large numbers of cocci in the peritoneal cavity, both extra- and intra-cellular, but many of the latter were liberated owing to phagolysis. Bacteriolysis was quite marked. The omental smear closely resembled that of the control mice and differed only in that a very occasional cell was found containing meningococci and these, together with a few rare cocci found free, might easily have been derived from the exposed surface of the omentum in making the smear. The possibility of migrating cells must not be overlooked in this connection. The striking feature was that phagolysis hardly existed and that cocci, to all intents and purposes, were absent.

Series C. The findings in this series were similar to those of series *A*, except that the majority of the cocci were intracellular and healthy looking cocci were fewer in number. Phagolysis and bacteriolysis were very marked.

Series D. The two mice which died in this series may be considered not to affect the experiment. One exhibited no cellular reaction whatever as judged by the peritoneal exudate and had comparatively few cells even in the omentum. The other was but little better. Both showed gram negative bacilli in the films.

Series E. In this experiment these mice were only examined macroscopically. The carmine was confined to the omentum and genital folds and only very little was found free in certain of the mice. Careful microscopical examination of many mice treated similarly to those of this series has been made, controlled by mice treated as in series *A*, when it has been found that the majority of the large numbers of cocci present in the peritoneal exudate are free and that the cells are chiefly encumbered with carmine. Although phagocytosis and bacteriolysis are evident, phagolysis appears to be less intense in the presence of the carmine. In the omentum cocci, cells and carmine are more abundant but their respective relations are those already described for the peritoneal exudate.

The surviving mice of this experiment were perfectly well when killed, 75 hours after inoculation, and presented the picture of recovered mice; healthy cells were the rule and aged polymorphonuclear leucocytes were being actively ingested by macrophages, and in an occasional one of these the remains of meningococci could be made out.

This and other experiments of the same nature (see Table 4) demonstrate very clearly that the apparent virulence of the meningococcus, when injected intraperitoneally, is greatly increased by interference with the functions of the phagocytes and omentum by means of carmine. At first sight it would appear that the protective influence of the omentum preponderates, but this is not the case, for a more critical examination shows:

(1) That such series as *B* and *E* are virtually identical owing to the phagocytes in the peritoneal exudate of the mice of series *B* being greatly encumbered with carmine.

(2) That although the minimal lethal dose has remained apparently unaltered in spite of the increased number of leucocytes in series C, there has been an enormous reduction in the numbers of free cocci compared with series A. Further, in other experiments than the one quoted, the "apparent virulence" has at times been reduced by the previous injection of broth, and at other times the mice have been killed very irregularly and so contrast strongly with the controls.

(3) That a very marked reduction in the apparent virulence is evidenced by series D in spite of the omentum being completely out of action in this series. These mice must be compared with those of series B and E, and it must be remembered, when considering this series, that, although the choking of the lymphatics and decreased surface for absorption may have played a part, the mice of series B were placed under identical conditions.

Thus it has been proved possible experimentally to increase the *apparent virulence* of the meningococcus by interference with phagocytosis and in this way to reproduce in the average mouse the condition that appeared to be responsible for increased susceptibility in the occasional mouse.

Further, by increasing the numbers of phagocytes present at the time of inoculation of living meningococci, it is possible to reproduce the opposite condition, that of apparent decrease in virulence of the culture.

It must not be supposed, however, that the measurement of successful phagocytosis or resistance to phagocytosis allows of the complete expression of virulence; something has already been said concerning the double effect *which bacteriolysis may produce, route and rate of absorbtion are open to* consideration and the importance of the localising influence of the peritoneal folds must also be remembered in the special case of intraperitoneal infection. These are only the more obvious factors involved in the study of the complicated subject of virulence and this determines the position that it is only the resultant of the opposing forces that can be measured and then only in terms of survival or death. Nevertheless, this and the preceding section demonstrate that the apparent virulence of the meningococcus, as determined by intraperitoneal inoculation of mice and rats, depends very largely upon resistance to phagocytosis and the bacteriolytic substances liberated by phagolysis.

VII. THE RAISING OF THE VIRULENCE OF THE MENINGOCOCCUS BY ANIMAL PASSAGE.

Pasteur's magnificent researches and their extension by other observers, have established that the virulence of many parasitic micro-organisms can be decreased at will by the application of appropriate methods. The process has been shown ordinarily to involve either singly or in combination, according to the nature of the parasite, the use of oxygen, light, heat, desiccation, chemical poisons, age or repeated sub-culture. Without entering into details, it will be admitted not to be beyond the bounds of possibility that these influences interfere with, or perhaps even suppress certain unknown important

physiological processes of the micro-organism which are assailing powers and thus the aforetime balance existing between parasite and host is upset. The complexity of this relation is demonstrated by the particular cases in which the virulence of certain parasitic bacteria is decreased for one species by raising it for another.

It is a matter of every-day experience to the immunologist and often a disconcerting one, that the maintenance of virulence of certain microbes is a matter of difficulty. Under such circumstances the usual practice is to resort to animal passage or constantly to procure freshly isolated strains.

In order that the method of animal passage may be applied it is essential to establish a minimal lethal dose and Dopter (1921, p. 85) summarises the general experience of bacteriologists with regard to the meningococcus as follows: "..., but nothing is so variable, on the whole, as the dose capable of causing death. One sometimes believes oneself to have grasped what one agrees to call the fatal dose of a particular strain, when a new experiment shows that animals, placed under identical conditions, resist, without one understanding why, a dose ten times as great as that previously established."

Such, too, was the writer's experience at the commencement of this investigation, but, with the gradual establishment of the conditions dealt with in Section II, this difficulty gave place to the possibility of being able to ascertain with certainty the minimal lethal dose of a given living culture, which remained constant so long as it was reasonably possible to exclude the influence of attenuating factors and the virulence was not accidentally raised. These conditions can be maintained for a week and even longer, as is shown in Table 1, but the ideal nutritive medium has not yet been found and consequently the influences of age and necessary sub-culture cannot be indefinitely excluded. Even were it possible constantly to obtain freshly isolated strains, there are, for certain purposes, decided advantages in knowing the properties of a selected strain, nor is every culture from human disease of standard virulence.

So it became important to investigate the conditions required for raising the virulence of the meningococcus. That such conditions exist for that organism, when subjected to animal passage, has been shown by Bruckner and Christeanu (1906) although Dopter and R. Koch (1909) and others admit complete failure.

In attempting to raise the killing power of the meningococcus by animal passage, considerable difficulty was experienced in recovering the micro-organism from mice which had succumbed to a dose of a culture of "low virulence"; nor was this difficulty much modified by resorting to cultures of "higher virulence," in which cases passage frequently succeeded at first but very soon the series was broken by failure to recover the meningococcus; and this obtained whether the method used was alternating cultivation and passage or direct transference of heart blood or peritoneal exudate from mouse to mouse. One factor contributing to this failure is the difficulty of maintaining an adequate dose of viable cocci, as indicated by the growth obtained from

the body fluids of dead mice. But what amounts to a mere difficulty in technique of dosage is not sufficient to explain why the killing power of a culture, recovered from a mouse or rat, is raised on one occasion and lowered on another, without exhibiting any definite sequence; nor can it account for the great variation in the number of viable organisms discoverable in animals even when a strain of high killing power has been used.

On the evidence described in foregoing sections it was assumed, as a working hypothesis, that the individual cocci composing a culture might vary considerably among themselves in the degree of their adaptation to parasitic existence and that the phagocytes and leukines might exercise a "selective" action. Under such circumstances, if the cocci of considerable parasitic capacity only survived, the maximum virulence of a strain would be obtained in a single passage; but should the resistance of the host be low, it would then be possible for the cocci of mean virulence for the particular culture to grow and as these are, presumably, in the majority the virulence of the strain recovered from the animal would be unchanged or even lowered by a single passage.

This hypothesis has been borne out by experiment. It has been possible to show that cultures recovered from the peritoneal cavity of animals (mice or rats), all of which received a fatal dose of a given culture at the same time, in certain cases had been raised in virulence as much as fourfold (incidentally as high a level as that strain has been known to reach), in other cases the virulence had remained stationary and in yet others it had been lowered.

Moreover, when mice have received a dose of carmine and meningococcus at the same time and the resistance of the host is thus hampered, in the manner already described, the apparent increase in virulence usually results in a more ready recovery of the strain from the animal, but, so far, no evidence has been forthcoming that the *actual* virulence of the strain has been raised. On the contrary, strains which have been recovered from mice which have been overwhelmed by the meningococcus, either by administering multiple minimal lethal doses, or by interference with the defensive mechanism of the animal, have usually shown a decrease in *actual* virulence.

Thus it is possible to raise the virulence of the meningococcus by animal passage, but the process is by no means straightforward and certain, on account of the uncontrollable variations in the defensive mechanism of the experimental animal. In point of fact, animal passage, as applied to the meningococcus, is essentially a matter of chance and consequently unsuitable for routine application in the production of therapeutic serums. The lack of suitable experimental animals is probably responsible for this condition.

VIII. The Raising of the Virulence of Meningococcus *in vitro*.

The accumulated evidence points significantly to the "selective" action of the phagocytes and liberated leukines being the determining factor in the raising of virulence of the meningococcus. Consideration of the fate of the

meningococcus inoculated intraperitoneally and the examination of strains recovered from animals so inoculated, demonstrate the uncontrollable variability of the factors contributing to what can be generally expressed as the resistance of individual experimental animals.

These various factors at present defy complete analysis; but, in the case of experimental intraperitoneal meningococcal infection with· purpose to raise the virulence of a strain, it is desired to realise:

(1) The conditions affording optimal physiological activity of the leucocytes and leukines.

(2) The conditions which enable the meningococcus to exercise its pathogenic properties only by the manifestation of its most complete physiological adaptation to a parasitic existence.

Occasionally these ideal conditions may be realised in a given experimental animal, but though it is possible to intensify the assailing powers of the meningococcus by animal passage, it is still beyond our powers to reproduce at will exactly the conditions required.

The conception that individual cocci composing a culture vary in their degree of adaptation to a parasitic existence suggested some experiments *in vitro* in which the leucocyte retains its role of "selective" agent. But because it is very difficult to maintain leucocytes under conditions ensuring their optimal physiological activity, the use of living leucocytes introduces as much uncontrollable variation as does the use of the whole living animal. Considering now, that when prevailing conditions cause very extensive phagolysis the range of action of any lysed cell is extended and that the concentration of leukines thus liberated may be such that it is immaterial whether the cocci are ingested or not, for they are then practically subjected to the conditions imposed by phagocytosis, extracts of leucocytes therefore were substituted for the living cells in these experiments.

Leukines are substances of unknown composition around which a vast literature has grown, which has been lucidly analysed by Levaditi (1914) and reveals the complexity of the experimental conditions it is desirable to realise.

The mode of extraction finally adopted by the writer was that described by Gengou (1921), because the substance obtained in this manner appears to be, almost certainly, that which produces bacteriolysis within the leucocytes themselves and its properties do not allow of its identification with either alexine or a proteolytic ferment. The cells used are the polymorphonuclear leucocytes of the rabbit and their extracts, made by Gengou's method, are remarkably bacteriolytic, as has been claimed for them by Gengou, and relatively small quantities will destroy vast masses of meningococci. That their action is non-specific and that immunisation does not produce a quantitative augmentation, the writer is able to confirm. The method used to collect the leucocytes appears to affect the potency of the extract obtained; this the writer measures in terms of the time required and the number of polymorphonuclears represented in the amount of extract which is sufficient to cause the

death of 0·0010 gm. of living meningococci, as determined by culture and not by the microscopical appearance of the cocci, using proper controls. It is surprising to notice the degree of change in the appearance of the cocci which is yet compatible with a relatively abundant growth. The most potent extracts have been obtained when the leucocytes have been collected by intrapleural injection of 20 c.c. of isotonic broth. A much greater yield is obtained by injecting a hypertonic solution containing 1 per cent. peptone and 1 per cent. to 2 per cent. of NaCl (see Bordet 1920, p. 183; Le Play and May, 1911), but in this case the extracts have been less powerful; the addition of potassium and calcium salts to the hypertonic solution produced no appreciable difference.

When hypertonic solutions are used the amount of fluid recovered, in 12 to 16 hours, from the pleural cavity of the rabbit exceeds the amount injected and when the cells have been removed, a transparent, yellow fluid remains which, as the writer's experiments show, possesses the properties of the Gengou extract of the washed cells and can be used in its stead.

When isotonic solutions are injected very little fluid remains after 16 hours and its properties have not been investigated, but the large amount of fluid remaining after seven hours has similar properties to that obtained with hypertonic solutions. It is found that the potency of extracts is not adequately expressed by the number of leucocytes represented by each c.c. of extract and it is desirable that further investigation be undertaken to determine the factors influencing this variation.

The independent variables controlling the reaction appear to be time, temperature, and relative concentration of the reacting substances, but the rule for their adjustment to obtain a desired result has not yet been determined and the present procedure is one of trial.

As both the culture and Gengou extract selected for a given experiment will vary respectively in degree of virulence and potency, compared with similar selections used in other experiments, the range of reaction in respect of time and relative concentration, when the temperature is kept constant (37° C. being the optimum), must be determined for any desired combination.

Although the Gengou extract alters but slowly when kept on ice, the fact that the properties of the culture change rapidly with age and sub-culture, ordains that only the shortest possible interval should intervene between the titration of the range of reaction and the attempt to recover a virulent strain from a given culture by the selective action of a Gengou extract; usually both stages of the experiment can be performed at one time.

The method at present employed is to select relative concentrations of the chosen culture and Gengou extract, such that the total mass of meningococci present is not destroyed within a time less than 48 hours, at 37° C.; the cultures obtained towards the end of that time have been found invariably to be more virulent than the original culture, or the control culture subjected to identical treatment only excepting the influence of Gengou extract.

The following experiment is given to show the method employed:

The weighed growth from a 20 hour culture, second generation from an egg culture, was emulsified in broth in a concentration of 0·0400 gm. living cocci per c.c.

An equal amount of this emulsion was placed in each of five tubes, to each of which was added the desired amount of Gengou extract, broth and 0·85 per cent. NaCl solution, such that each contained an identical concentration of broth, excepting tube 2 which only contained 60 per cent. of the desired amount because of the volume of extract it was desired to add.

The relative concentrations of the reacting substances together with the results of culture are shown in Table 3.

It must be stated that the leucocyte extract used in this experiment is the most powerful the writer has made and it has only been equalled by two others.

Table 3.

Titration of Potency of Gengou Extract.

Tube No.	Living meningo. in gms. per c.c. of mixture	Amount of Gengou extract in c.c. per 0·0010 gm. meningo.	Growth on sub-culture after contact at 37° C. for the time indicated in hours							
			0	6	23	27	48	72	120	144
1	0·0080	0	+ + +	+ +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +
2	0·0080	0·08 (=72 × 10^4 polymorph)	+ + +	+	1 col.	0				
3	0·0080	0·04 (=36 × 10^4 polymorph)	+ + +	+ +	+ +	+	0			
4	0·0080	0·02 (=18 × 10^4 polymorph)	+ + +	+ +	+ +	+	+ +	+	0	0
5	0·0080	0·01 (=9 × 10^4 polymorph)	+ + +	+ +	+ + +	+ +	+ +	+ +	+	0

+ + + = Very good growth.   + + = Good growth.   + = Poor growth.   0 = No growth.

The microscopic changes were followed closely at the same intervals of time as the viability, but it is not thought necessary to burden the paper with these long descriptions, although they are a helpful guide and of some interest.

At this stage an 18 hour culture, the second generation from the same egg culture which provided the growth for the above titration, was divided into aliquot parts: (1) the first part was inoculated into mice, (2) the second was subjected to the action of the above Gengou extract, in a concentration of 0·03 c.c. (= approximately 27×10^4 polymorphs) per 0·0010 gm. of living meningo., (3) and the third portion was placed under identical conditions as (2) but substituting 0·85 per cent. NaCl solution for the Gengou extract. The second and third mixtures were then placed at 37° C.

This concentration of extract was chosen as it was estimated that the total mass of cocci would not be destroyed in less than 80 hours, thus allowing time for the multiplication of such cocci as would survive the antagonism of the extract.

Under these conditions the culture obtained after 75 hours' contact was selected for animal inoculation with the results given in Table 4.

Table 4.

(Each dose was given to two mice.)

Toxin	Series	Living meningo. in gms. per 20 gms. of mouse	Carmine in gms. at same time as coccus	Result
Culture untreated with Gengou extract	1	0·0160	0	Both died
		0·0080	0	One died
		0·0040	0	One died
	2	0·0080	0·005	Both died
		0·0040	0·005	One died
Culture after 75 hours' treatment with Gengou extract	3	0·0040	0	Both died
		0·0020	0	Both died
		0·0010	0	One died
	4	0·0040	0·005	Both died
		0·0020	0·005	Both died
		0·0010	0·005	Both died

Considering only the mice which did not receive carmine (series 1 and 3), it is evident that the minimal lethal dose for the strain of meningococcus used has been raised from 16 mgms. to 2 mgms.; that is to say that before treatment with Gengou extract the amount of this strain which constituted one minimal lethal dose was 1/1250 part of the body weight of the mouse and after the virulence was raised, in the manner described, the same result was obtained by a dose equivalent to 1/10,000 of the body weight of the animal. This represents a very considerable increase in virulence for the meningococcus. In other experiments the time required has been shortened by varying the concentrations.

The writer has never yet failed to raise the virulence of a culture by this method, but the degree to which it has been raised varies because the exact conditions required have not been determined. So far as present experimental evidence goes it seems to indicate that increased concentration of potent extract, with conjugate shortening of the viable period, imposes such conditions that a given culture of low virulence does not attain to as high a degree of killing power as it does when the conditions are less exacting. One point of importance respecting this statement must be emphasised: namely, that in order to obtain a marked increase in virulence the extract used must be strongly bacteriolytic; it is expedient to employ a low concentration of a strong extract rather than a high concentration of a weak one.

It is incorrect to suppose that it is sufficient merely to place meningococci in contact with an extract of leucocytes, and, at some time before the complete extermination of the cocci, to recover a culture which will be raised in virulence, as if it were simply a matter of destruction of the weaklings and survival of individual cocci of full assailing power pre-existing in the original culture. That this is not the state of affairs has been indicated, time and again, by the fact that cultures recovered from contact with a high concentration of potent

extract, have failed to reach the degree of virulence attained by the same strain in the same experiment subjected to the influence of a very much lower concentration of the same leucocyte extract. In fact in certain experiments the cultures recovered from contact with a high concentration of extract have actually shown a diminution in killing power.

This point is illustrated by the following experiment chosen as a typical example from among several others.

A 20 hours' growth, second generation from an egg culture, was emulsified in 0·85 per cent. NaCl solution in a concentration of 0·0200 gm. of living cocci per c.c. Part of this emulsion was inoculated into mice and the intraperitoneal minimal lethal dose proved to be 0·0080 gm. of cocci for 20 gms. of mouse. The remainder was equally distributed between three tubes each containing 0·25 c.c. of broth for each milligramme of cocci added. The final volume was adjusted so that the concentration of cocci was 0·0010 gm. per c.c., by adding physiological saline alone to tube A, and Gengou extract and physiological saline to the other two, so that tube B contained 0·5 c.c. of extract (equivalent to $51·7 \times 10^5$ polymorphs) and tube C 0·1 c.c. of extract to each milligramme of cocci. These three tubes were then incubated at 37° C. for 42 hours and the sub-cultures grown from each of them at that stage were emulsified in 0·85 per cent. NaCl solution and injected intraperitoneally into mice. The minimal lethal dose of these cultures for 20 gms. of mouse proved to be 0·0080 gm. of cocci for tube A, 0·0040 gm. for tube B, and 0·0020 gm. for tube C.

The culture used in this experiment was one of low virulence and the extract was rather on the weak side; the concentrations used were determined by previous experiments.

It appears to the writer that the reaction is partly controlled by a delicate balance of antagonistic factors resulting in an adaptation on the part of the living cocci of an evolutionary nature. When it is required to raise the virulence of a strain, the writer sets up a series of varying concentrations and follows their progress by cultural and microscopical methods, and periodical animal experiment.

No evidence has been obtained to indicate that the leucocytes of immunised animals yield an extract exhibiting a greater or less degree of efficiency than those of normal animals. A point of general interest is raised by the fact that extract of rabbit leucocytes are capable of raising the virulence of meningococci for mice and rats.

It must be mentioned here, that, although fresh normal guinea-pig serum exhibits an extremely marked bactericidal action on the meningococcus, the writer has not succeeded in raising the virulence of that organism by means of such serum; although it is also true that the virulence of the cultures used was not lowered by the use of such serum.

Specific bacteriolytic sera have not, so far, been used by the writer. When hypertonic solutions have been injected into rabbits, the fluid recovered, freed of cells by centrifuging, has given identical results to those obtained with

leucocyte extracts of moderate potency; this property survives even after the fluid has been heated at 55° C. for 30 minutes.

One more observation must be mentioned as it might confuse the issue if not recognised. It is frequently found that the control tube, containing no Gengou extract, gives cultures which show a slight increase in virulence compared with the original culture. The degree of virulence attained is very seldom comparable to that reached by cultures from tubes in which the correct concentration of extract obtains, nor is it constant or progressive, but fluctuates. In some ways it resembles the influence of sub-culture, previously discussed, and it is probably closely connected with certain factors influencing virulence which will be discussed in another paper.

The convenience of this *in vitro* method of raising the virulence of the meningococcus, the degree of control that can be exercised over the conditions of experiment, and the rapidity and certainty with which the desired result is obtained, constitutes, in the writer's opinion, a decided advance on the method of animal passage. Many points remain which require experimental investigation and their interest and practical importance is undeniable.

The writer has refrained from discussing the time factor in detail because its influence is still under investigation. But that does not detract from its importance and it is desired to emphasise it, by stating that when cocci are placed in contact with a suitable concentration of leucocyte extract to yield cultures of enhanced killing power, the virulence is raised progressively with time; but the time factor appears to have a limiting value, for with excessive action a slight reversion of virulence has been observed. The properties of leucocyte extracts and details of the methods for their preparation will not be discussed as the references to the literature amply cover the subject, to which but little has been added by the writer.

IX. The Influence of Desiccation on Virulence.

It has long been recognised that drying in air, at room temperature or at 37° C., on cotton wool, filter paper, or other porous substance is detrimental to the life of the meningococcus (Dopter, 1921, p. 81; Vines 1916, p. 13; Treadgold, 1916, p. 14) and it is claimed that under these conditions, whether in the form of cultures or contained in mucus, the meningococcus dies in 6 to 12 hours. Reference has already been made to the fact that desiccation over H_2SO_4 *in vacuo* is rapidly fatal to the meningococcus and this emphasises the interest in the method described by Swift (1921) for preserving cultures of various organisms, including the meningococcus, depending upon drying the frozen growth over P_2O_5 and glycerine at very low pressures. (It has long been shown that the meningococcus survives temperatures as low as $-20°$ C., Dopter, 1921.)

It must be emphasised that these apparent contradictions are evidence that the observations are incomplete, so that the general law determining the influence of desiccation on the meningococcus has not been formulated.

Drying on swabs and materials exposed to the air at variable temperatures involve many uncontrolled factors and for experimental purposes the method has, therefore, a limited application. When attention is directed to desiccation over H_2SO_4 *in vacuo* at 37° C., one factor, which is not generally recognised, must be taken into account: that is the vapour pressure of H_2SO_4. This matter first attracted the writer's attention, when working with dysentery bacilli during 1916–19, owing to the relatively copious deposits of sulphates on the walls and platform of the desiccator and the sides of vessels containing the large masses of growth under investigation from which ammonia or amines were constantly liberated. The writer is inclined to blame this appreciable partial pressure of H_2SO_4 for the death of the meningococcus under such conditions, because that organism is readily recovered from growth which has been dried, until its weight becomes constant, over NaOH or P_2O_5 at 40 mm. Hg pressure and at 37° C. These are special cases, but they serve to show that the meningococcus readily survives desiccation, without recourse to the elaborate technique of Swift, and their application to further study of the question of virulence is of interest.

When a given culture of meningococcus is killed by desiccation over H_2SO_4, the minimal lethal dose bears the same relation to that of the living culture as does the minimal lethal dose of cocci killed by heat; in both cases a very much larger dose of the dead cocci is required to kill; as much as twenty times the dose of living cocci may be necessary. This statement holds good even for those strains of which the minimal lethal dose of extracted endotoxin represents a smaller mass of cocci than does the minimal lethal dose of living cocci.

When, however, the cocci have been dried to constant weight over NaOH or P_2O_5, *in vacuo* at 37° C. or by Swift's method, the cultures recovered from the desiccate have not altered in virulence. Furthermore, when the desiccate itself is emulsified and inoculated into animals it kills in exactly the same dose as did an aliquot part of the growth used for desiccation, and cultures of meningococci can be recovered from the mice.

Thus it appears that the assailing factors possessed by a virulent strain of meningococcus are not interfered with by even so drastic a measure as desiccation, by which between 80 and 84 per cent. by weight of the original growth has been removed in the form of moisture and gases, so long as the viability of the cocci has not been destroyed.

A more extended generalisation than this cannot at present be justified, because only strains of moderate virulence have been tested and sufficient time is required to discover the influence of age upon the desiccate.

X. DISCUSSION.

(a) *On the minimal lethal dose.*

It is claimed, in this paper, that it is reasonably possible to establish a minimal lethal dose for a given culture of meningococcus, which is constant

over a sufficient period to enable the experimental investigation of virulence to be undertaken. There are defects, only too easily discerned, in the method, but reference to the literature will allow of the concession that a small step has been taken in the right direction. The most obstinate impediment, though, perhaps, not the most obvious but certainly the most important, are the involved questions of the physical conditions, the nutrition and the removal of waste products of cultures, which are essential conditions for the maintenance of the complete physiological activity of the parasite. Questions relating to this part of the problem, the relation of virulence to medium have been omitted purposely from this paper, with the intention of dealing with them separately.

(b) *On virulence.*

Gordon (1920, p. 22–25) demonstrated that killing by ether, heat and desiccation destroyed the pathogenicity of the meningococcus although (p. 43) none of these agents destroy its contained endotoxin. His desiccation experiments are open to the objection that he etherised his material before drying it, even though, in the method he used, the ether boils off *in vacuo* at 37° C., and is absorbed by the H_2SO_4. Nevertheless it is true that desiccation at 37° C. *in vacuo* over H_2SO_4 does destroy the pathogenicity of the meningococcus when used as *entire* cocci; even in those particular strains which, weight for weight of the same culture, kill 20 gms. of mouse in a much smaller dose when completely broken up than they do when living. This property, thus easily lost by the meningococcus, Gordon called "the labile factor" and he expressed disbelief that it simply depends upon the vital capacity of the coccus, as expressed by growth *in vitro*. Gordon sought without success to discover its origin in a soluble toxin, a haemolytic substance and a ferment, but he showed very clearly that the killing power decreased in proportion to the diminution in number of viable cocci in a given culture. The evidence now brought forward in this paper amply confirms Gordon's far seeing deduction, although no closer approximation is possible than to state that the property is a function of the viability of the organism, partially expressed by its power to resist the mechanism involved in the cellular defence of the host.

Tulloch (1920, pp. 79–81) was unable to obtain evidence, by *in vitro* experiments, that thermolabile antiphagocytic substances are developed by the meningococcus in its growth; but he did observe that the susceptibility of the meningococcus to phagocytosis varied in different cultures. Further, he was unable to obtain cultures which would give consistent results over periods longer than three or four days; but he does not state whether liability to phagocytosis increased with sub-culture. It is noteworthy that he observed no difference between living cocci and those heated at 60° C. for 30 minutes, particularly as this does not conform with observed phagocytosis *in vivo*. It would appear that either or both of two difficult conditions were uncontrolled, namely the "virulence" of the organism and the optimal conditions for the leucocytes to function.

The apparent virulence of the meningococcus can be increased or diminished by appropriate interference with the defensive mechanism of the host. For this purpose substances which are inert in respect of the vitality and integrity of the leucocytes and bacteria must be used, such as isotonic broth and washed carmine, and not an inoffensive microbe such as *B. subtilis* (*B. prodigiosus* was used by Dopter (1921, p. 86)), because the writer has observed that *B. subtilis* is capable of liberating the endotoxin of the meningococcus and dysentery bacillus apparently unaltered and also is capable of living in the peritoneal cavity of mice and even producing fatal results. Dopter also failed to bring about a like result by the use of leucotoxic serums; perhaps this was due to the liberation of leukines (see Bordet, 1920, p. 348; Yoshinaga, quoted by Levaditi, 1914, p. 489).

It appears that the evidence of the literature, when taken in conjunction with that produced in this paper, suggests strongly that the capacity of the meningococcus to invade the living tissues rests upon an undefined property, depending upon the life of the coccus, which can be expressed by resistance to phagocytosis. But additional evidence has been produced that this alone is not sufficient to express completely the degree of virulence of the culture, which is not simply a property peculiar to the parasite, but depends upon a balance of a variety of factors contributed by both host and parasite.

The degree of virulence is measured by the intensity of the factors contributed by the parasite which is just sufficient to overcome the intensity of the factors contributed by the host and can be expressed in terms of the mass of parasite required to bring about the death of the host. But this minimal lethal dose is an unsatisfactory "unit" because of its liability to variation with small derangements of the contributing factors, whether originating in host or parasite, which cannot at present be estimated.

For the present this measurement of virulence must suffice, and, with the recognition of its shortcomings, it can be made to serve a useful purpose in the study of variation in virulence due to alteration in factors contributed by the parasite.

(c) *On the raising of the virulence of the meningococcus.*

Very few authors claim to have raised the virulence of the meningococcus; Bruckner and Christeanu (1906) did so by intraperitoneal passage in rabbits and Ruppel claims to do it by a process which he refused to divulge (see Dopter, 1921, p. 425), but Dopter and other acknowledged authorities did not succeed in their various attempts. Up to the present no very precise analysis has been made of the conditions which it is essential to observe, and, in view of the general admission of failure in respect of the meningococcus, the writer feels that the contribution set forth in this paper is a step towards bridging this gap in our knowledge.

The fact that in the presence of a high concentration of Gengou extract a given culture does not yield so virulent a strain as it does when subjected to

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a lower concentration, with conjugate lengthening of the viable period, suggests that the raising of virulence is not merely a selection of pre-existing resistant individual cocci, but, rather, an appropriate balance of factors, which affords the optimal conditions for the survival and multiplication only of such organisms as are capable of developing physiological properties tending to endow them with a more complete adaptation to parasitic existence.

The convenience, rapidity and unfailing success of the *in vitro* method described, are its greatest recommendations, although it is not without theoretical interest and perhaps it may prove capable of general application by appropriate use of various types of cells.

It is a matter of interest that Alexander (1918), working with pneumococci, found that both sensitised cocci and cocci incubated at 37° C. for 6–8 hours with leucocytes were too virulent to use in large intravenous doses to immunise rabbits; but, that sensitised cocci incubated with leucocytes were attenuated though they did not lose their vitality.

XI. Conclusions.

1. It is possible to establish an intraperitoneal minimal lethal dose for living meningococcus of any strain, which remains constant over a period determined by the rate of loss of "virulence" of the culture consequent on its growth *in vitro*.

2. The minimal lethal dose of a culture so determined and expressed in terms of the mass of living cocci which is just sufficient to kill "unit" weight of experimental animal of average resistance for the species, is an incomplete expression of virulence; but it is a sufficiently close approximation to render it possible to appreciate certain factors contributing to virulence and to study their individual variation and influence.

3. Virulence is not a property of the parasite alone, but is the resultant of physiological forces exerted by both parasite and host. Variation in any factor contributed by either participant materially affects the experimental measurement of this resultant, so that an *apparent* increase or decrease of virulence is readily effected.

4. Resistance by the meningococcus to the mechanism and processes of phagocytosis, is the expression of undefined properties peculiar to the parasite contributing to virulence. This resisting power of the parasite depends upon it

being alive and becomes more evident as the amount constituting the minimal lethal dose decreases in mass.

5. It is possible to increase or decrease the virulence of a strain by animal passage, but the extent and direction of the change cannot be predicted in any experiment, because the determining factors are at present beyond complete analysis and control in the living experimental animal.

6. The virulence of a given culture of meningococcus can be raised with certainty *in vitro* by subjecting it to the influence of a suitable extract of polymorphonuclear leucocytes. The amount of change produced depends upon temperature, time and the relative concentration of the active agents.

An optimum exists for each of these variables: that for temperature (37° C.) is dependent upon the very nature of the leucocyte extract, whilst those for time and relative concentration are dependent upon one another and are determined by the nature of the reaction and the activity of the reacting agents.

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