Discussion.

Most of the avirulent variants produced a high degree of resistance in sheep and two of them, as judged by the tests, gave a solid immunity. The successful variants were those that showed the highest protective power in guinea pigs. Avirulent dissociants can be isolated easily and rapidly, so that for practical purposes only those giving sound results in guinea pigs need be tried on sheep. Nevertheless, even strains which produced a relatively poor immunity in guinea pigs were comparatively successful when tried on sheep.

However, considerable variation existed between the degrees of immunity produced by the different strains. The cause is unknown but the conditions under which the variants can be obtained are as yet ill-defined and the problem may be solved by a refinement in technique and a more rigid standardisation of the medium. This is the more likely because differences in growth were noted with different batches of media and sera.

The immunity produced by the avirulent dissociants was compared with that given by two batches of an ordinary vaccine strain (Pasteur II type). This gave as good an immunity as the best of the roughs. However, this Pasteur type vaccine was exceptional in that it elicited a better immunity than any similar vaccine prepared here during many years. Moreover, its virulence was on the borderline of safety. The experiments were slightly weighted against the avirulent strains as far as dosage was concerned.

The experiment with strain XXII A_2 noted in table LXXIX introduced an interesting possibility. The usual dose of this strain did not produce a strong immunity in guinea pigs or sheep, but when ten times this concentration was given the sheep acquired a solid immunity, although the glycerine-saline suspension of strain XXII A_2 was then

four months old.

The tenfold strength contained about 3,000,000 spores per dose and it is quite feasible to manufacture vaccines containing this number of organisms. Therefore, it appears probable that larger doses of potent avirulent strains may give maximal immunity to anthrax. It was shown (table XII) that large doses of the ordinary vaccines improve immunity, but this increased dosage carries with it an increased risk, whereas almost any dose of the avirulent strains can be tolerated.

The immunity tests with the avirulent rough variants must be considered very satisfactory and indicate, as far as laboratory experiments can, that domestic animals can be easily and safely immunised against anthrax with avirulent strains. This is particularly important in goats, horses and wild animals in zoological gardens (Neitz, 1936). These animals are highly susceptible to the use of any but weak, poorly immunising Pasteur I type vaccines, which means that these animals are practically non-immunisable. Potent avirulent strains should prove very useful in such cases. In laboratory tests not set down here, one C.K.D. of strain XXVIII killed almost every goat immunised with a Pasteur I type vaccine, whereas it was shown in section A that a relatively poorly immunising avirulent strain could produce a fair immunity in goats.

SUMMARY.

(1) Virulent anthrax strains grew mucoid and produced avirulent rough dissociants on serum agar in a number of different carbon dioxide concentrations.

(2) A relatively small dose of avirulent spores immunised sheep against anthrax.

(3) The immunising power of the avirulent rough strains was not associated with the carbon dioxide concentration in which they developed.

G.
DISSOCIATION OF ATTENUATED ANTHRAX STRAINS ON 50 PER CENT SERUM AGAR IN 65 PER CENT CARBON DIOXIDE.

In the early part of this work (Parts A - C) it appeared as if rough avirulent variants derived from attenuated or avirulent smooth mucoid strains were not efficient immunising agents. In the last part of the work (Parts D and F) it was shown that avirulent rough dissociants, obtained from virulent strains growing smooth mucoid in carbon dioxide, were very efficient immunisers. To test the possibility of correlation between the degree of virulence of the parent strain and the degree of immunising ability of its avirulent dissociant, the procedure of part D was applied to relatively attenuated anthrax strains. That is, they were grown on serum agar in carbon dioxide.

Three strains were used: the first was isolated from a commercial saponin vaccine and was avirulent for rabbits but killed about half the number of guinea pigs inoculated with it. The second was one of the laboratory vaccine strains of about the same degree of virulence. The third strain was also isolated from a saponin vaccine but was considerably more virulent, although it did not kill rabbits. All three strains were intermediate rough-smooth and produced a slightly mucoid growth on nutrient agar in air. Their colony morphology was typical of vaccine strains in ordinary use.

Each strain was streaked onto the surface of 50 per cent serum agar and incubated in 65 per cent carbon dioxide at 37°C. All three strains were smooth mucoid after 24 hours growth. They were examined daily and the smooth mucoid growth spread steadily, until at the end of eight days the smooth mucoid growth almost covered the surface of the medium. As there was no hope of obtaining variants in this way, the experiment was discontinued.

Now and then during the experiment, small cuneiform patches were seen at the edge of the colony, but these were covered by smooth mucoid material and could not be separated from it. There was little chance for the commencing rough variants to establish themselves as they were almost immediately swamped by the more rapidly growing smooth mucoid part of the strain. With virulent strains rough variants appeared early and multiplied more rapidly than the smooth mucoid part of the culture so that they could establish themselves and even encroach on the smooth growth. In the case of the attenuated cultures the smooth mucoid variant grew more rapidly and the rough dissociants were not able to establish themselves. Moreover, the rate of dissociation seemed faster in the virulent than in the attenuated strains.

III.

DISCUSSION.

In sections A and B of this paper experiments are described where a number of anthrax strains were subjected to lengthy periods of ageing and intermittent growth to obtain smooth or smooth mucoid variants. Five virulent strains, one partly attenuated and one much attenuated strain were used. The first study was made on virulent Boshoff and this strain eventually developed smooth mucoid colonies which were virulent for guinea pigs and rabbits. There was considerable difficulty in maintaining this strain without making subcultures every one to two days, and this difficulty proved due to the rapid production of fast growing rough variants, which overgrew the parent mucoid colony. The smooth mucoid strain continuously threw off clearly defined rough variants, and the same sequence of events occurred after each new subculture and even after subcultures from single cells. Apart from the difference in colony type, the rough dissociant differed

from the smooth mucoid parent in its inability to produce capsules in carbon dioxide or in the animal body; and although the smooth mucoid strain was fully virulent for guinea pigs, the rough variant was not able to kill mice. Nevertheless, in spite of its avirulence, the rough variant produced a considerable resistance to anthrax in guinea pigs, sheep and goats.

If it be assumed that a smooth mucoid anthrax strain is analagous with the smooth "normal" form met with in other bacteria, then its behaviour bears a close resemblance to the classical S - R variation accompanied by a loss of virulence found in the majority of pathogenic bacteria. It is unusual, however, for avirulent roughs to possess any marked immunising power, and Basset (1935) states that avirulent strains do not immunise against anthrax. However, de Kruif (1921), Cowan (1923), Korobovka and Kotelnikov (1935) and Felix and Pitt (1935) found avirulent immunising variants of Pasteurella, streptococci and salmonella, and Bail (1915) described a similar phenomenon in B.anthracis. Therefore, the observations made in the present work are not unique.

In the particular case of the Boshoff strain the abrupt transition from smoothness to roughness, and from virulence to avirulence, was accompanied by the loss of the power to develop capsules. Anthrax septicaemia and smooth mucoid colony form are both invariably associated with the capsuled bacillary form, so it is very likely that the loss of the capsule was the determining feature of the change of Boshoff to the rough and to the avirulent state. It is unwise to press the analogy with other pathogenic bacteria too far, as S - R variation is not always determined by capsule loss, although equivalent phenomena have been described in streptococci (Dawson and Olmstead 1935) in B. Friedlander (Julianelle 1926), in pneumocci and in other organisms. Nevertheless, the clear cut picture of dissociation shown

by the Boshoff strain and the very promising immunity conferred by the rough variant encouraged further experimentation and accordingly four more virulent strains and two attenuated strains were examined in experiments similar to those conducted with V. Boshoff.

From virulent Anthrax A a smooth mucoid strain was obtained which seemed morphologically identical with that found in V. Boshoff and showed the same type of continuous dissociation. However, the smooth mucoid variant was unable to kill guinea pigs, but was virulent for mice. The rough dissociants from the smooth mucoid growth were quite devoid of virulence. Neither variant conferred a detectable immunity on guinea pigs or mice. From virulent Drummond a smooth mucoid strain developed which dissociated less readily than the strains already described, but which too gave rise to some rough variants. Neither variant was virulent for guinea pigs; but the smooth mucoid strain killed mice, whereas the rough was quite avirulent. Neither variant immunised guinea pigs.

Strain 568 was attenuated in broth at 42°C until plates showed a mixture of smooth and rough colonies. Each type was subcultured until the smooth became slightly mucoid. Neither variant was virulent for guinea pigs or mice, nor did either variant produce any immunity in guinea pigs.

Strain IV was grown in broth at 42°C until subcultures showed some slightly smooth mucoid colonies. Later subcultures tended to be smooth. Occasionally, the smooth growth showed rough variants, but these were slightly mixed with smooth elements. The smooth variant was virulent for guinea pigs, while the rough killed only one out of six guinea pigs. This rough conferred a fairly good immunity on guinea pigs, although the protection was not equal to that given by the Boshoff strain.

Virulent Pretoria North gave rise to smooth mucoid

colonies after prolonged growth in serum. These very rarely dissociated, but eventually some rough colonies were isolated. The smooth mucoid culture was barely virulent for guinea pigs and mice, while the rough was quite avirulent. The rough strain produced a high degree of resistance to anthrax in guinea pigs, and the protection was equal to that conferred by the avirulent rough Boshoff.

Virulent strain 568 was grown in phenol broth until a smooth mucoid colony was obtained. Dissociation was very rarely seen, but eventually a rather mixed rough variant was separated. Neither variant killed guinea pigs, although both variants were slightly virulent for mice. Neither variant immunised guinea pigs.

Thus the ease with which smooth mucoid strains could be obtained varied considerably, but the procedure in the case of virulent strains was always lengthy.

Once the smooth mucoid or smooth strains had been got, all showed the S - R dissociation. The rate and the extent of this change varied considerably in the different strains. The Boshoff and Anthrax A mucoids dissociated rapidly and continuously, whereas the virulent 568 mucoid dissociated very rarely and the variants in this case were not well defined. Nevertheless there were two points of general agreement amongst all the strains tested: firstly the S - R change was associated with a more or less complete loss of the ability to grow capsules, and secondly there was a loss of virulence with the shift from the SM to the R form. In the case of strain 568, the variants were about equal in virulence, but as was noted at the time the two variants were not well defined morphologically. For the rest, where a difference in virulence existed the rough variant was always found to be the less virulent.

There were indications that the power of the rough variants to immunise might be a function of the virulence

of the smooth mucoid parent strain. The Pretoria North strain was, however, an exception, but when another attempt was made to examine this strain the smooth mucoid characteristic had been lost. However, there was no doubt that the ability of the avirulent variant from Boshoff to immunise was not an exceptional case, since the rough variants from Strain IV and Pretoria North were also able to increase the resistance of guinea pigs.

The practical importance of avirulent strains possessing a high degree of immunising power is very great, provided such strains can be obtained with regularity. The methods already discussed did not yield constant results, and were neither simple nor reasonably quick. The loss of the capsule with the SM - R change may be of use in the study of dissociation as the capsule is a very easily recognised morphological feature.

From the point of view of colony dissociation in general, the results obtained were rather unusual. When the smooth mucoid strains had been obtained they appeared to dissociate in the classical manner. That is S - R, accompanied by a reduction or complete loss of virulence. However, it is well known that fully virulent anthrax strains are very rough, so that dissociation in anthrax appeared to proceed thus: R (virulent) - SM (virulent or avirulent) R (avirulent). The SM - R change seemed typical of dissociative changes generally, but the R (virulent) - SM change was completely reversible and probably depended on factors other than genetic modifications: as, for example, in the reversible S - R change in the typhoid organism grown at 30°C (Felix, Bhatnager and Pitt, 1934). This aspect of the problem was discussed fully in the introduction to section C, and for the reasons stated there the dissociation of virulent strains in carbon dioxide was attempted. It seemed likely that more information on the role of the capsule might be obtained, and the possibility existed

that if dissociative processes of virulent strains gave rise directly to avirulent roughs, these might possess a higher immunising value than those derived from smooth mucoid strains of reduced virulence.

Dissociation of virulent strains in CO_2 :-

Three rough virulent strains, Pretoria North, Drummond and 568 were grown under a partial pressure of carbon dioxide, and all strains developed more or less marked smooth mucoid characteristics. The Drummond strain became almost as mucoid as a typical smooth mucoid strain, while 568 showed only a slight tendency to develop this type of growth. All these strains showed some rough variants after seven to fourteen days. When transplatations from the smooth mucoid and from the rough growth were made onto agar in air, the resulting cultures showed no differences in colonial morphology, but in two of the strains the subcultures from the smooth mucoid growth in carbon dioxde were definitely more virulent than the subcultures from the rough variants. In the case of strain 568 the results were less definite but tended in the same direction. None of the strains yielded a completely avirulent rough, and although the rough variants were not capsuled in the cultures, they became capsuled in the animal. This meant that although the carbon dioxide favoured capsulation it was not in this respect the equivalent of *in vivo* conditions. Thus the rough colony form in carbon dioxide was not an index of complete avirulence, although it indicated reduced virulence. The procedure was also unsatisfactory in that the dissociation rate was slow, and the strains were subjected to a moderately lengthy period of growth before they could be examined. Again, however, the rough dissociants were significantly less virulent than the smooth parent strains, but in this case the variants could only be distinguished, morphologically, when grown under the special conditions of the experiment.

The results obtained were encouraging and confirmed the impression gained in the earlier part of the work. Accordingly, virulent strains were grown on serum agar in carbon dioxide to see if they would develop more fully mucoid characteristics.

Dissociation of virulent strains on serum agar in CO_2 :- Seven virulent strains were grown on 50 per cent serum agar in 65 per cent carbon dioxide. Six of the strains were fairly recently isolated and three of these were tested immediately after isolation: only one strain had been obtained a long time before. The s trains were markedly smooth mucoid when examined after 24 hours, and all seven showed a great readiness to produce rough uncapsuled variants in the carbon dioxide as early as 24 hours after inoculation. There was very little difficulty in picking and transplanting these rough variants, as they were sharply demarcated from the original smooth mucoid growth. Transplants from the smooth mucoid growth in carbon dioxide onto ordinary agar in air showed an immediate reversion to the typical very rough growth of the original virulent strains and the same applied to transplants from th rough variants. Therefore these two variants so clearly to be differentiated on serum agar in carbon dioxide, were indistinguishable from one another when grown under ordinary conditions. If the two variants were again grown on serum agar in carbon dioxide, the one again resumed the dissociating smooth mucoid state, while the other was again rough and devoid of capsules. On testing the pathogenicity of the variants from the different strains the subcultures from the smooth mucoid variants were nearly all virulent, while the subcultures from the rough variants proved to be mostly avirulent. The virulence tests were done with transplants grown in air and all these were very rough; so that although the colony morphology of the two variants was

identical under these conditions, yet there was a sharp difference in pathogenicity between the cultures originating from the smooth mucoid growth and those originating in the rough variants on serum agar in carbon dioxide. The protocols (Section D) indicate that the completely avirulent rough variant was not always got at the first attempt. It is readily conceivable that the selection of rough variants from a mixed culture must sometimes result in the carrying over of smooth material. However, rough avirulent strains were obtained in all seven cases without much difficulty, so their production must be frequent if not usual. Where the rough variant still killed, it grew mucoid when transplanted onto serum in carbon dioxide.

Now these rough avirulent strains all arose from virulent anthrax strains after a very short period of growth and most of them conferred a high degree of immunity on guinea pigs. The immunity was of a much higher order than that produced with the variants obtained by the earlier methods; so much so indeed that one injection of these latterly isolated variants gave as good an immunity as two injections of the previously isolated strains. Moreover the immunity produced by the strains isolated in carbon dioxide was tested with strains more virulent than those previously used. The degree of immunity conferred by the different avirulent rough strains obtained in carbon dioxide varied; but the differences were not marked, and all these avirulent roughs effectively immunised guinea pigs. One strain which had given good results in guinea pigs, was tried on sheep, and the immunity produced was far better than that given by the ordinary vaccine used. The two strains were compared under identical conditions. (Strain XVIII, table LIII). The least effective of the avirulent strains was also tried on sheep and gave better results than were expected.

Another five virulent strains were grown in

different carbon dioxide pressures (Section F) and rough avirulent variants were found to develop over a wide range of carbon dioxide concentrations. However, there appeared to be an optimum concentration in the neighbourhood of 30 per cent. Extensive tests on sheep showed that the majority of the avirulent strains produced a very satisfactory immunity. Even more important was the fact that an increase in dosage, within reasonable limits, markedly improved the immunity produced by a relatively poor strain.

This method of growing virulent strains on serum in carbon dioxide rapidly and consistently yielded avirulent rough variants having the power of conferring immunity to anthrax. From the practical standpoint the method is important because the immunity appeared to be of a higher order than that produced by the usual attenuated vaccine strains. The difficulty of protecting guinea pigs and rabbits with ordinary vaccines is well known although rabbits have sometimes been immunised to small test doses. Guinea pigs immunised with Pasteur I strains have shown very little resistance to less attenuated strains. Recently, Mazzucchi (1934), Rammon and Staub (1935) and others have claimed to increase the resistance of rabbits by adding either saponin or lanolin to the inoculum, to limit the rate of absorption of the organisms. However the test doses used were not large. Grajewski (1935) failed to immunise rabbits with several doses of "carbozoo" vaccine, and Hruska (1934) could not immunise rabbits unless a fairly large number died of the vaccine itself. In the present work, the guinea pig was used because its uniform susceptibility to anthrax lessened the experimental error. For the same reason doses of bacilli far in excess of those usually employed to test the immunity of small animals were used. The results show that the rough avirulent strains appeared to possess a greater immunising power than the ordinary vaccine strains.

It seems also that the avirulence of these strains

should make anthrax immunisation a safe procedure. Some of the variants have been used in the experimental work over a long period and have remained completely innocuous. It is difficult to reverse dissociative changes so that it is likely that the variants will remain avirulent. Munne (1934) stated categorically that capsule formation and multiplication at the site of injection were essential for the production of immunity to anthrax. However, capsule production is not essential but it is possible that capsules increase the survival time of the bacilli in the animal. In that case the uncapsuled variants will not be as effective immunisers as can be expected from the experimental work where large doses were used. Against this argument stand the results obtained with sheep (Sections D and F) where small doses of the avirulent strains were used for immunisation.

Sporulation and stability are other factors of importance in vaccine preparation. These properties were not influenced by the dissociation. The sporulation and the stability of the avirulent roughs (although not specifically mentioned in the protocols) appeared to be functions of the degree to which the original virulent strain possessed these properties. Avirulent mutants from freshly isolated and vigorously sporulating virulent strains sporulated vigorously, and this property was as stable in the avirulent dissociant as in the original virulent strain. Continued rapid subculturing affects sporulation adversely, and this applies as much to the virulent parent strain as to the non-virulent variant. It was easier to obtain vigorously sporulating avirulent roughs than to obtain vigorously sporulating strains by the usual methods of attenuation. The avirulent roughs obtained in sections D and F sporulated like fully virulent strains. This was probably due to the rapidity with which these avirulent strains were obtained from freshly isolated virulent strains.

The ease with which the avirulent immunisng variants

were obtained from the virulent strains makes the questions of sporulation and stability less important. Moreover the work of Elser, Thomas and Steffen (1935) and of Flosdorf and Mudd (1935) on the preservation of biological products by drying in vacuo from the frozen state may very well solve the problem of conserving the properties of various strains. It would have been interesting to see if there was some relation between the immunising power of a rough avirulent strain and the virulence of the strain from which it originated. Attempt to obtain rough dissociants from attenuated strains grown on serum agar in carbon dioxide failed because although these strains grew very smooth mucoid they were so stable that no rough variants could be obtained. In this respect the attenuated strains behaved altogether differently from the virulent strains. This rather interesting observation was not investigated further.

The results obtained in sections D and F indicate, as far as laboratory experiments can, that domestic animals can be easily and safely immunised with avirulent strains. This is particularly important in goats, horses and wild animals in zoological gardens (Neitz, 1936). These animals are highly susceptible to the use of any but very weak, poorly immunising pasteur I type vaccines, which means that these animals are practically non-immunisable. Potent avirulent strains should prove very useful for these animals. In laboratory tests not quoted in this paper one C.K.D. of strain XXVIII (section F) killed almost all goats treated with the pasteur I type goat vaccine, whereas it was shown that the relatively weak avirulent strains from Boshoff produced a fair immunity in goats (tables IX and X).

General aspects of S - R variation in anthrax:

From the point of view of a dissociation problem the experiments described in this paper show some very interesting features. The normal virulent anthrax strain is very rough, and in the ordinary course smooth mucoid strains appear only

in old or in attenuated strains. Some of these mucoid strains were shown to vary continuously (Boshoff and Anthrax A) and the dissociant in these cases was rough, non-mucoid, uncapsuled and avirulent. In this respect the smooth mucoid anthrax strains resembled the "normal" group of other pathogens. When, however, virulent strains were grown under specified conditions they immediately became smooth mucoid and showed a dissociation picture identical with that shown by strains such as the normally smooth mucoid Boshoff or Anthrax A strains. This smooth mucoid growth shown by virulent strains on serum agar in carbon dioxide was not a permanent change and was immediately reversible when the ordinary conditions of aerobic growth were restored. The same process occurred with single cell isolations of virulent strains so that the change to smooth mucoid and back to rough was conditioned by the environmental change and was not due to a selective action on particular cells, nor to a dissociative change.

It is probable that the "normal" appearance of virulent anthrax is smooth mucoid, since the mucoid bacillus and the in vivo bacillus are both invariably capsuled. Therefore, the appearance of virulent anthrax strains grown on serum agar in carbon dioxide may very well be the normal appearance of the virulent strain. Thus the loss of capsules and of smoothness on the ordinary laboratory media are possibly due to an "unfavourable environment". The dissociation into avirulent roughs in carbon dioxide appears to be a true dissociation and in this particular case the change is associated with the loss of ability to form capsules. At least, in all cases where the S - R change with loss of virulence has taken place, the capsule loss has been the constant feature and it is unlikely that this should be merely coincidence.

The role of the capsule in anthrax has interested

many experimenters. It is universally known that animal anthrax bacilli are all capsuled. On the other hand many weakly virulent or quite avirulent strains are smooth mucoid and fully capsuled, so that the virulence must be independent of the capsule production, or alternatively, the capsules produced by virulent bacilli must be different from those produced by attenuated or avirulent bacilli. Morphologically there is no difference between the capsules seen in anthrax septicaemia, the capsules from virulent strains in carbon dioxide, and the capsules from avirulent smooth mucoid strains. Chemically and immunologically all capsules appear to be the same (Tomcsik and Bodon, 1934). Thus the capsules from virulent or avirulent strains cannot be distinguished by any known method. However, if the capsule is not associated with the virulence of the anthrax bacillus, it would be difficult to explain the results obtained by Bail, and the results obtained in the present work where the loss of the capsule is closely associated with the S - R virulent to avirulent mutation.

Any theory of immunity and attenuation in Anthrax must include the following observations and must offer a reasonable explanation for them.

(1) The fact that vaccines attenuated at 42°C retain their ability to produce capsules, but lose virulence, and that the greater the loss of virulence the less the immunity produced.

(2) Virulent strains which have suddenly lost the ability to produce capsules (by mutation) are immediately avirulent, but these completely innocuous strains possess a greater immunising power than quite virulent vaccine strains attenuated at 42°C.

It is immediately apparent (from (1)) that the capsule cannot be a unique factor determining virulence, although (from (2)) it must be of great importance. Secondly

there is nothing to show that the capsule is at all concerned in theproduction of immunity. All virulent strains are, however, able to produce capsules, although the converse does not hold.

These rather conflicting observations can be reconciled if a special factor determining virulence is assumed. Such a factor or virulence (Vi) antigen was described in S.typhi by Felix, Bhatnagar and Pitt (1934) and has been confirmed by a number of other workers. Kauffman (1935), Robertson and Yu (1936). The Vi antigen was demonstrated serologically and shown to be intimately associated with the resistance of the bacterium to phagocytosis. Before this time it was known that only smooth strains of typhoid were virulent, but there was no explanation for the wide divergences in virulence between smooth strains. However, the discovery of the Vi antigen clarified the situation. According to Felix and Pitt (1935) a fully virulent strain must have its complete quota of Vi antigen and must be smooth - that is, possess the smooth somatic (O) antigen.

If neither Vi nor O are present then the strain is avirulent, rough and non-immunising.

If Vi but not O are present then the strain is avirulent and rough, but has immunising properties.

Let it be assumed for the moment (1) that the virulence of an anthrax strain is determined by an antigen called the virulence or Vi antigen; (2) that the immunity to anthrax produced by any strain is due to this same antigen.

According to this scheme two factors must be considered: the virulence antigen and the capsule. The variations of virulence and immunising power in anthrax strains might then occur as follows:-

(1) The virulence antigen may decrease until none is left, while the faculty of producing capsules

remains intact. Such a process will lead to a graded diminution in virulence until the strain becomes avirulent and at the same time the immunising power will grow less. This is a description of what occurs, for example, in strains attenuated at 42°C. These strains gradually lose virulence and with this immunising power while the ability to produce capsules remains unimpaired.

(2) The virulence antigen may remain intact and the strain lose the ability to develop capsules. This will result in an immediate and complete loss of virulence; but the immunising power should be of a high order. The avirulent rough strains isolated on serum agar in carbon dioxide appear to be examples of this process. It may also happen that the virulence antigen is reduced and the strain is unable to produce capsules. Such variants will be avirulent, but the immunising power will decrease as the Vi antigen decreases. A strain may thus become devoid of Vi antigen and ability to form capsules. This will then be avirulent and non-immunising. Several variants of this type were isolated in the early part of the work.

(3) Another possibility is for a strain to lose the ability to produce capsules gradully, but to retain the Vi antigen. Such a strain may eventually show slight virulence, but good immunising power. The Pretoria North strain isolated early in this work (Section A) may be an example. This strain however was the only one encountered where slight virulence of the smooth variant was associated with good immunising properties, and there may be other explanations for this.

(4) A strain may suddenly lose the ability to produce capsules. This may happen at any time and could occur during other processes of attenuation and also in relatively attenuated strains. Such strains would suddenly become avirulent. The immunising power should depend on

the amount of Vi antigen remaining and in general this should be less than that present in virulent strains. The strains would then be avirulent and have immunising power proportional to the virulence of the parent strain. As shown in the earli part of the discussion, there is evidence that the immunising power of the rough avirulent variants does depend on the virulence of the smooth mucoid from which they arose. It is also possible that the rarely encountered avirulent vaccine strains possessing slight immunising power may arise in this way.

It also seems as if changes in virulence which are not referable to loss of the capsule are usually gradual; as for example in ageing strains or during attenuation. The highest degree of immunising power would probably be possessed by uncapsuled variants from very deadly strains of anthrax, provided that the uncapsuled variant could be obtained quickly by methods which do not greatly affect the "Vi antigen The method of dissociation on serum agar in carbon dioxide appears promising.

The above statement is no more than a working hypothesis which has proved useful in the present work and may prove useful in further work. It offers an interpretation of a number of observations and is reasonably economical in the number of assumptions made. The term virulence or Vi has been borrowed from the work of Felix and his co-workers. The present investigations on anthrax paralleled the work with typhoid in many respects and there seems to be an analogy between the relationship of Vi to O antigen in typhoid and the capsule and hypothetical Vi antigen in anthrax. The analogy exists mainly in the possibility of assigning the factor for virulence and immunity to one antigen. The direct proof of the existence of a Vi antigen in anthrax is lacking.

It was shown in sections D and F that virulent anthrax strains dissociate in an apparently orderly manner from the time they are isolated. In the particular cases investigated, there was a continuous variation in the direction of uncapsuled and avirulent types. The regularity of the process makes it difficult not to entertain the hypothesis that this may be a constant feature of growth in anthrax, even under natural conditions, and that it may have a bearing on epidemiological and immunological problems in nature.

An interesting aspect of the investigation was the variation in the dissociation rates of the different virulent strains. This carries the possibility that there are fundamental differences between virulent strains, and that these differences may play a determining role in, for example, the persistance of an infection in the field.

After the completion of the major part of this work my attention was drawn to a paper by Stamatin (1934) where he described a smooth mucoid to rough variation, with loss of virulence, in smooth mucoid strains of anthrax. In 1936 Stamatin and Stamatin tested one of these strains on rabbits and obtained a fair immunity. Schaeffer (1936) found that virulent strains were mucoid when grown on coagulated serum. He obtained some rough variants and found that after a number of generations on serum they were reduced in virulence or even rendered avirulent. At the same time the ability to produce capsules was lost. Sometimes these roughs still killed and produced capsules. Schaeffer, therefore, postulated two kinds of rough, stable and unstable. This assumption seems unnecessary in view of the difficulty of ensuring absolute homogenicity in selecting variants. This difficulty occurred to some extent in the present work, and the use of coagulated serum rendered

Schaeffer's work particularly liable to this source of error. Inspissated serum was tried in the earlier part of the present work without marked success. Fully virulent strains did not grow very mucoid and moreover they exhibited such marked proteolytic power that the surface of the serum was liquified and this rendered the isolation of variants exceedingly difficult (Section D, page 64).

IV.

SUMMARY AND CONCLUSIONS.

1. Smooth mucoid colonies developed in anthrax cultures held for long periods at 37°C in a number of different media. Similar colonies developed in strains attenuated at 42°C.

2. All these smooth mucoid colonies yielded rough variants which were usually less virulent than the smooth parent and which in some cases showed a complete and abrupt loss of virulence as compared with the parent strain.

3. The loss of virulence and smoothness was associated with a loss of the ability to produce capsules both *in vitro* and *in vivo*.

4. A number of these rough avirulent daughter strains conferred immunity to anthrax on guinea pigs.

5. Fully virulent and freshly isolated anthrax strains always grew smooth mucoid on serum agar in carbon dioxide.

6. These virulent strains rapidly developed rough daughter colonies in the carbon dioxide, in the same way that attenuated smooth mucoid strains developed rough daughters under ordinary conditions (summary 1 and 2).

7. These rough variants obtained on serum agar in carbon dioxide were completely avirulent and uncapsuled *in vitro* and *in vivo*.

8. Most of these rough avirulent dissociants were

able to produce a high degree of immunity in guinea pigs and sheep.

9. Evidence is brought forward concerning the significance of the capsule in virulence and immunity.

10. The above findings are discussed.

ACKNOWLEDGEMENTS.

My thanks are due to Dr. E. M. Robinson, the Chief of the Section, and to Dr. J. H. Mason for advice and assistance during the course of the work.

V.

REFERENCES.

ARKWRIGHT, J.A. (1920). Variation in bacteria in relation to agglutination by salts and by specific sera. Journ. Path. & Bact. Vol. 23. pp. 358-60.

ARKWRIGHT, J.A. (1921). Variation in bacteria in relation to agglutination both by salts and by specific sera. Journ. of Path. & Bact. Vol. 24. pp.36-40.

BAERTHLEIN, K. (1918). Ueber bakterielle Variabilität, insbesondere sogenannte Bakterienmutationen. Zentralbl. f. Bakt. I. orig. Vol. 81. 6. pp.369-435.

BAIL, O. (1915). Veränderung der Bakterien im Tierkörper. Ueber die Korrelation zwischen Kapselbildung, Sporenbildung und Infektiosität des Milzbrandbacillus. Centralbl. f. Bakt. Paras. u. Infekt. Krank. orig. Vol. 75. pp.159-173.

BAIL, O. (1915). Veränderung von Bakterien im Tierkörper. Untersuchungen ueber Kapsellosen Milzbrand. Ibid. Vol. 76. pp.38-46.

BAIL, O. (1915). Veränderung von Bakterien im Tierkörper. Abschwächungsversuche am Milzbrandbacillus bei 42°C. Ibid. Vol. 76. pp. 330-342.

BASSET, J. (1936). Essais sur L'immunité. P. 36. Vigot Fréres, Paris.

COWAN, (1922). Variation phenomena in streptococci, with special r eference to colony form, haemolysin production and virulence.
Brit. Journ. Exp. Path. Vol. 3. pp.187-195.

COWAN, (1923). Variantion phenomena in streptococci. Further studies on virulence and immunity.
Brit. Journ. Exp. Path. Vol. 4. pp.241-246.

DAWSON, M.H. and OLMSTEAD, M. (1934). The mucoid phase of streptococcus haemolyticus.
Science. Vol. 80. No. 2074. pp.296-297.

DE KRUIF, P.H. (1921). Dissociation of microbic species. Coexistence of individuals of different degrees of virulence in cultures of the Bacillus of Rabbit Septicaemia.
Journ. of Exp. Med. Vol. 33. pp.773-789.

DESKOWITZ, M. and SHAPIRO, A. (1935). Numerical relations of an unstable variant of salmonella aertrycke.
Proc. Soc. Exp. Biol. and Med. Vol.32..No.4. pp.573-577.

ELSER, W.J., THOMAS, R.A., and STEFFEN, G.J. (1935). Dessication of sera and other biological products (including microorganisms) in the frozen state with the preservation of the original properties of the products so treated.
Journ. of Immun. Vol. 28. No. 6. pp.433-473.

FELIX, A., BHATNAGAR, S.S. and PITT, R.M. (1934). Observations on the properties of the Vi antigen of B. typhosus.
Brit.Journ. of Expt. Path. Vol.15, No.6. pp.346-354.

FELIX, A. and PITT, R.M. (1935). The virulence and immunogenic activities of B. typhosus in relation to its antigenic constituents.
Journ. of Hyg. Vol. 35. No. 3. pp.428-436.

FLOSDORF, E.W. and MUDD, S. (1935). Procedure and apparatus

for preservation in "lyophile" form of serum and other biological substances.

Journ. of Immun. Vol. 29. No. 5. pp.389-425.

GRAJEWSKI, W. (1935). Le controlé de laboratoire de vaccin charbonneux "Carbozoo" de Mazzucchi.

Rev. Veter. Slave. Vol. 2 No. 1. pp.17-18.

GRATIA, A. (1924). Variations microbiennes et infection charbonneuse.

Compt. Rend. Soc. Biol. Vol. 90. pp.369-371.

HRUSKA, K. (1934). Der Rolle der Glukoside bei der Impfung mit virulenten Bakterien Milzbrand Glukosidkultur.

Zeit. f. immun. Forsch. Vol. 81. pp. 367-376.

JULIANELLE, L.A. (1926). Immunological relationships of encapsulated and capsule-free strains of Encapsulatus pneumoniae. (Friedländers Bacillus).

Journ. Exp. Med. Vol. 44. pp.683-695.

KAUFFMAN, F. (1935). Ueber einen neuen serologischen Formenwechsel der Typhusbacillen.

Zeits. f. Hyg. u. Infekt. Vol. 116. No.6. pp.617-651.

KOROBOVKA and KOTELNIKOV. Mikrob. Epid. et Paras. Vol. 14.

MASON, J.H. (1933). A new culture tube.

Journ. South Afr. Med. Assoc. Vol. 4. No.2. pp.89-90.

MASSINI, R. (1907). Arch. f. Hyg. 61. p. 250.

MAZZUCCHI, M. (1934). Le charbon hématique.

12th Intern. Veter. Congr. Vol. 2. pp.138-156.

MUNNE, V. (1934). Les nouvelles méthodes de vaccination anticharbonneuse.

12th Intern. Veter. Congr. Vol. 2. pp.157-167.

v.NAEGELI, C. (1877). Untersuchungen über die niedere Pilze und ihren Beziehung zu den Infektionskrankheiten und der Gesundheitspflege.

NEITZ, W.O. (1936). Anthrax: Death in Blesbuck following the use of Goat Anthrax Vaccine.

Journ. South Afr. Vet. Med. Assoc. Vol. 7. 3. pp.119-120.

NUNGESTER, W.J. (1929). Dissociation of B. anthracis.
Journ. infect. Dis. Vol. 44. No. 2 pp.73-125.

RAMON, G. and STAUB, A. (1935). Essais sur la vaccination charbonneuse.
C. Rend. Soc. Biol. Vol. 119. No. 25. pp.1073-1076.

REIMANN, H.A. (1925). Variations in specificity and virulence of pneumococci during growth in vitro.
Journ. Exp. Med. Vol. 41, 5. pp.587-600.

ROBERTSON, R.C. and YU, H. (1936). The Vi antigen of B. typhosus.
Journ. Path. and Bact. Vol. 43. (1). pp.191-196.

SCHAEFFER, W. (1936). Dissociation de la Bacteridie charbonneuse.
C.R.Soc. Biol. Vol. 122. 25. pp.1178-1181.

STAMATIN, N. (1934). Contributions a l'etude de la morphologie et la biologie de la Bacteridie Charbonneuse
Arrhiva Veterinara. Vol. 26. 1/2 pp. 1-28.

STAMATIN, N. and STAMATIN, L. (1936). Le pouvoir immunisant de souches acapsulogénes de Bacillus anthracis.
C.R. Soc. Biol. Vol. 122. 19. pp.491-495.

TCHAIKOWSKY, W.K. (1935). Methoden zur Gewinnung einer einheitlichen Milzbrandvakzine und Abgetöteten Kulturen zur Impfung.
Zentralbl. f. Bakt. u. infekt. Krankh. orig. Vol. 134. No. 3/4. pp.239-244.

TODD, E.W. (1928). Further observations on the virulence of haemolytic streptococci with special reference to the morphology of the colonies.
Brit. Journ. Exp. Path. Vol. 9. 1. pp.1 - 6.

TOMCSIK, J. and BODON, G. (1934). Ueber die serologische identität der Kapselsubstanz von auf Nährboden gezüchteten und tierischen Milzbrandbazillen.
Zeitschr. f. Immun. Forsch. Vol. 83. No.5/6. pp.426-7.

WAGNER, G. (1920). Beitrage zur Kenntnis der Milzbrand- und

Milzbrandähnlichen Bazillen.

Centralbl. f. Bakt. I. orig. Vol. 84. pp. 386-[illegible].

WEBSTER, L.T. (1925). Biology of Bacterium leptisepticum. Effects of oxygen tension and the presence of rabbit blood on growth, dissociation and virulence.

Journ. Exp. Med. Vol. 41. 4. pp.571-585.