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The Use of Saponin Spore Vaccine for Inoculation against Anthrax in South Africa.

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INTRODUCTION.

THE use, for immunization, of anthrax strains suspended in a solution of saponin or digitonin was first recorded by Hruska (1931). He showed that a rather virulent Pasteur II strain suspended in five per cent. saponin could be safely injected into rabbits, and that these rabbits would subsequently withstand the injection of a virulent strain. Non-immune rabbits, moreover, proved resistant to injections of virulent anthrax bacilli suspended in ten per cent. saponin. The same worker (1932) again claimed that a virulent strain suspended in saponin was not dangerous to rabbits. He experimented with different concentrations of saponin (1 per cent.—10 per cent.), and with different doses (0·05 c.c.—0·5 c.c.), and concluded that the concentration and amount of saponin per dose were far more important than the number of bacilli. He also said that safety was directly proportional to the amount of local reaction and necrosis. Thus it seemed that 0·5 c.c. of a suspension of virulent bacilli in ten per cent. saponin was safer than 0·05 c.c. of the same suspension, although the former contained ten times as many bacilli as the latter. Nevertheless the small number of rabbits used, and the meagre differences between the results with different concentrations, do not warrant definite conclusions. In these experiments, a number of rabbits inoculated with virulent bacilli suspended in different amounts of saponin died. This hardly bears out Hruska's contention that rabbits can be safely inoculated with virulent bacilli in saponin.

In 1931, two horses, two bovines, and two goats, withstood an injection of a virulent strain in saponin, and these animals subsequently proved immune to anthrax. Rabbits that survived a similar inoculation were also immune. Later work (1934a) showed that the longer saponin acted the less dangerous virulent spores became. There are, however, indications—in Hruska's tables—that the immunity elicited by such suspensions was poorer than the immunity produced by younger suspensions. It should also be noted that the tables given in Hruska's paper were selected from a large number of observations, so that it is difficult to assess the results accurately.

Encouraged by these results, Hruska initiated field experiments on a small scale, and, in 1933, Weidlich published the results of immunizing 3,000 cattle with Hruska's saponin vaccine. This vaccine was a suspension of a Pasteur II vaccine in four per cent. saponin, because Hruska considered the practical difficulties of using virulent strains too great (danger to human beings and uninoculated animals), although no evidence had been adduced to show that a virulent suspension could be used in field work. Weidlich considered the results better than those obtained with the Pasteur method, but said that swellings and systemic reactions were sometimes alarming, and milk yields sometimes seriously impaired. It should be noted that, according to Hruska's method of preparation, three Roux bottles make 2,000 doses of vaccine. As anthrax vaccines go this is a rather dense suspension.

Weidlich (1934) and Hruska (1934b) described a fairly extensive test in Carpathia where 70,000 head were inoculated with a Pasteur II strain in one per cent. saponin (note that the saponin concentration has been further reduced) with very satisfactory results. About 0·3 per cent. of the animals immunized with saponin vaccine died subsequently of anthrax, whereas about 1·5 per cent. of those immunized by Pasteur's method, and 3·0 per cent. of uninoculated animals died. Hruska said that none of the animals inoculated with his vaccine died as a result of the inoculation. The superiority of Hruska's vaccine seemed evident and the results were encouraging, although the tests, which were spread over a number of districts, were not strictly comparable.

Shortly after the publication of Hruska's first paper (1931) on the effect of saponin on anthrax strains, Mazzucchi, of the Istituto Sieroterapico Milanese (I.S.M.), stated that his "Carbozoo" vaccine (1929a, 1929b, 1930, 1931) consisted of a *virulent* anthrax strain suspended in ten per cent. saponin; and later Santarelli (1932) mentioned that "Carbozoo" contained a virulent strain in two per cent. saponin. To our knowledge, no further details of the constitution or manufacture of this vaccine have ever been divulged by the I.S.M. Mazzucchi (1929a) said that "Carbozoo", which as he emphasized, contained a virulent strain, was almost innocuous for guinea-pigs and harmless for rabbits. One injection was said to immunize rabbits and sheep solidly, and about 75 per cent. of goats. Guinea-pigs showed an increased resistance to anthrax after inoculation with "Carbozoo", but were not immune.

The total number of animals used in these experiments were 1,000 guinea-pigs, 100 rabbits, 30 goats, and 200 sheep. No details were given of the allocation of the animals to the different experiments—experiments which covered a very wide field—and the total number of controls for the larger animals was five goats and eight sheep. A more detailed publication by the I.S.M. would be of great interest.

Belfanti (1929) and Alessandrini (1929) discussed a limited number of field experiments carried out by the latter in which about 600 cattle were inoculated with "Carbozoo". Twelve animals showed extensive swellings, but recovered, and a further two died of anthrax, although these deaths were not considered a

result of the inoculation. Amongst the 600 head were 150 dairy cattle. None of these aborted, nor was there any reduction in milk yield. In a small experiment with 22 sheep, Alessandrini showed that " Carbozoo " produced a better immunity than the Pasteur vaccine. Mazzucchi (1930 and 1931) inoculated about 500,000 head of mixed stock, and got excellent results. No breakdown of immunity occurred (no indication given of degree of natural infection), and only two of the inoculated beasts showed swellings. Both recovered and, from these two cases, Mazzucchi deduced that swellings produced by ordinary vaccines were fatal, while those produced by " Carbozoo " were not.

These publications initiated a great deal of research on saponin anthrax vaccines, and while some workers were able, wholly or in part, to confirm the work of Hruska and of Mazzucchi, others reported undesirable sequelae to inoculation; and others again were quite unable to get satisfactory results with the new vaccine.

Venturi (1931) inoculated 41,600 head of mixed stock with " Carbozoo " and said that local reactions were quite marked. One goat died three days after inoculation, and milk yields were slightly affected.

Anreiter (1932) controlled a severe outbreak of anthrax in Upper Austria with saponin vaccine, after Sobernheim's method had proved disappointing (partly owing to a shortage of serum). Some cows lost their milk, one horse had to be treated with serum, and a goat died of vaccine anthrax. One occurrence of anthrax in an inoculated ox was considered a natural infection. In all, 14,000 head received Mazzucchi's vaccine and the results were good.

Troger (1932) tested " Carbozoo ", which, Belfanti told him, consisted of fully virulent spores in saponin, on rabbits. Thirty six animals received 0·125 c.c. to 0·75 c.c. and two died. The smears and cultures were positive for anthrax, but Troger attributed the deaths to coccidiosis.

Gerlach (1931, 1932) discussed the outbreak described by Anreiter (1932) and said that of 15,000 cattle inoculated with " Carbozoo " eleven died. These deaths might have been a result of the inoculation, but this possibility could only be confirmed in two animals.

Voyáček (1932) stated that a dense suspension of virulent organisms in 1:40 digitonin or 1:10 saponin, in serum-saline, did not kill guinea-pigs in doses of 0·1 c.c.

Bory (1933) found " Carbozoo " avirulent for horses and cattle, but moderately virulent for rabbits. He did not believe Mazzucchi's vaccine contained virulent spores, and considered the strain resembled the Pasteur strain. Although " Carbozoo " immunized sheep more satisfactorily than the double Pasteur vaccine, Bory advised caution, because he thought the new vaccine might prove rather virulent. He also stated that a vaccine strain suspended in saponin was less virulent and produced a better immunity than the same strain without saponin.

Hall (1934) in Nigeria tested a Pasteur II strain in two per cent. saponin. Thirty-two sheep and twenty goats survived an inoculation of this vaccine, and all but one sheep and three goats resisted a virulent dose given later. This vaccine contained up to one thousand million spores per dose: even weak vaccines would, in such concentrations, give a satisfactory immunity. Hall said that this strain, if used without saponin, often killed goats.

Eichhorn and Lyon (1934) in America used a number II spore suspension in two per cent. saponin and got excellent results. At first they used five per cent. saponin, but this caused rather severe reactions. Lyon said that the strain used was of American origin, and that the vaccine could be used for horses as well as for other animals. It was further claimed that the addition of saponin resulted in a better immunity, apart from making the vaccine safer. As all the tests published in this paper were done under laboratory conditions, it is difficult to see how the higher margin of safety was assessed.

Hupbauer and Gojkovic (1935, 1936) found the ordinary Pasteur vaccine too virulent, and therefore prepared a glucoside vaccine containing a large number (400 million) spores per dose. This was tried on 12,000 cattle and sheep, none of which died. The immunity was said to last eight months, as a rule; but only four months in heavily infected districts. Severe local and systemic reactions sometimes followed inoculation.

Megyaszai (1936) inoculated 200,000 sheep, 70,000 cattle, and 3,000 horses and confirmed, in general, the value of saponin vaccine. He stated, however, that severe reactions sometimes occurred in dairy cattle and in working oxen, and that the vaccine was unsuitable for horses.

Gerlach (1936) reaffirmed his earlier statements (1931, 1932) concerning the success of saponin vaccines, but pointed out that horses often developed cachexia, and sometimes died, after inoculation. Gerlach now recommended 0·5 to 1·0 per cent. saponin.

Lopez (1937), in Spain, reported that he had successfully used a Pasteur II strain in one per cent. saponin on about a million cattle. Unfortunately the detailed results are not yet available.

The workers quoted above have, broadly speaking, confirmed some of the claims of Hruska and of Mazzucchi. It seems established that saponin vaccines have been more satisfactory than the Pasteur vaccine or the Sobernheim. Many workers have reported some undesirable reactions with the saponin vaccine, and many have said it should not be used for horses, although the discoverers claimed complete safety for all domestic animals. Voyáček is the only worker, apart from Hruska and Mazzucchi, to claim that virulent strains can be made innocuous by adding glucoside. Only Bory has compared the effects of the same strain with and without saponin. Unfortunately, we have only been able to see abstracts of these two papers.

The authors referred to below are those who have not found saponin vaccine so successful.

Staub (1932) reported that Hruska's vaccine (saponin and Pasteur II) could kill rabbits, and he also obtained the rather anomalous result that this relatively virulent strain, with saponin, produced a far poorer immunity in rabbits than a double intradermal inoculation of Pasteur vaccine. Staub got the same result when he made up his own saponin vaccine, although he used quite a dense suspension (60 million spores per c.c.). He concluded that saponin reduced virulence, but did not affect the production of immunity. Ramon and Staub (1936) found that the immunization of sheep and rabbits with saponin vaccine was less successful than with the double Pasteur, the spores plus lanolin, or the Besredka methods.

Jezić (1933) was unable to substantiate Hruska's work on the innocuity of virulent strains in ten per cent. saponin, and also found that adding saponin to weak vaccine strains did not improve them. Jezić concluded that the efficiency of glucoside vaccines depended on the saponin and on the potency of the strain used.

Huber (1933), in Batavia, was also unable to make a suspension of virulent spores safe by adding saponin. In one experiment, seven of nine sheep died of anthrax after inoculation of a saponin vaccine prepared from a virulent strain. Huber also incorporated Hruska's own strain in saponin, according to the directions of the discoverer, and found that the vaccine, although producing good immunity in sheep, caused quite severe reactions. In another experiment, this strain, in digitonin, killed five of six goats. Huber also compared the immunity produced in goats by a fairly dense suspension of Hruska's strain in digitonin with that elicited by an ordinary single spore vaccine made from a vaccine strain obtained from Onderstepoort. (In South Africa this strain is used exclusively to immunize goats and equines.) Both the digitonin vaccine and the plain spore vaccine produced an excellent immunity, but the former caused severe reactions. Huber considered it too dangerous for field use.

Manley (1934), in Cyprus, carried out several experiments with a virulent strain suspended in saponin. At first he suspended the strain in ten per cent. saponin and found that rabbits and large animals, although surviving an inoculation of this vaccine, showed serious local lesions. Belfanti (of the I.S.M.) then advised Manley to use two per cent. saponin, but although the immunity produced by this suspension was good, the reactions, which included the death of a goat, were severe; and precluded the use of this vaccine in the field. An ordinary spore vaccine, made from an Onderstepoort strain, gave as good an immunity as the saponin vaccine. It must be pointed out that a rabbit inoculated with Manley's virulent strain, without saponin, survived; so this strain cannot be reckoned fully virulent.

Zovijefski (1935) found that a vaccine made according to Hruska's prescription gave a good immunity, but was dangerous to horses.

Grajewski (1935) failed to immunize rabbits with several inoculations of " Carbozoo " vaccine. He also said Mazzucchi's vaccine was equivalent to a Cienkowski II; that is, virulent for guinea-pigs, but not for rabbits.

Terentiev (1937), in the U.S.S.R., inoculated a number of horses, bovines, sheep, and goats, with a Zenkovsky II vaccine in both 2·5 per cent. and 10·0 per cent. saponin. It seemed as if the animals inoculated with the spores in 2·5 per cent. saponin developed the better immunity. All sheep inoculated with a virulent strain in ten per cent. saponin died.

Discussion of Literature.

1. *Constitution of saponin vaccines.*—To our knowledge, Mazzucchi has always claimed that the strain he uses is virulent. Both Grajewski and Bory, however, could only isolate attenuated strains from "Carbozoo", and neither Jezić, Huber, nor Terentiev could make a virulent strain innocuous by adding saponin. Voyáček claimed that a virulent strain in saponin-serum-saline did not kill guinea-pigs. Unfortunately we have only seen an abstract of this paper, but suggest that the anthracidal effect of serum may have played a rôle. Manley, also, was able to make a virulent strain non-lethal to sheep, but in this case there is evidence showing that the strain used was not fully virulent. The reactions were, however, very severe. Hruska, Zovijevski, Hall, Eichhorn and Lyon, Hubbauer and Gojkovic, all used *attenuated* strains in their vaccines and, apart from the claims by the Italian workers, there is no evidence of virulent strains ever having been used in field inoculations. Huber considered even Hruska's attenuated strain (Pasteur II), suspended in saponin, too virulent for large scale use. It is also clear, from Hruska's and Weidlich's publications, that Hruska's earlier vaccines were stronger and more prone to cause reactions that the later products.

There has also been considerable speculation about the concentration of saponin that should be used. Hruska, in his earlier papers, maintained that the safety and efficacy of his vaccine depended, to a marked extent, on the presence of a fairly large amount of saponin. Nevertheless this does not seem to have been of such critical importance, because, in successive years, Hruska reduced the glucoside concentration from ten to four and, finally, to one per cent. Gerlach said, later, that 0·5 per cent. could be used. Hall, Eichhorn and Lyon, Lopez *et al*, all used one to two per cent. saponin.

On the whole, therefore, it seems unlikely that virulent strains can be used in vaccines, and it is also unlikely that "Carbozoo" contains a virulent strain.

2. *Efficacy of saponin vaccines.*—Most workers, except Staub, and Ramon and Staub, seem agreed that saponin vaccines have proved better than some older types of vaccines (e.g. the double Pasteur; Sobernheim's). Kind (1922), Viljoen, Curzon and Fourie (1928), Quin (1929), Huber (1933), Manley (1934), have shown, however, that single spore vaccines can give excellent results. Therefore saponin vaccines, before a sound evaluation of their worth can be made, should be compared with good single spore vaccines. Unfortunately, little work has been done along these lines; and it is perhaps significant that saponin vaccine has found most favour in

countries that had not, up to the time of adopting saponin vaccine, used good spore vaccines. In America, for example, the quality of anthrax prophylactics was, prior to the introduction of " Carbozoo ", very poor. Gochenour *et al* (1935) tested a large number of American anthrax prophylactics. These workers made the entirely wrong statement *that one certain killing dose* of virulent anthrax spores would always kill a proportion of animals, however solidly these were immunized. They therefore employed in their tests a virulent suspension that would not kill all uninoculated controls. Even when measured by such a weak standard, most of the vaccines tested gave meagre or no protection. Apart from saponin vaccine, almost any good spore vaccine would have been a great improvement.

Huber, in Batavia, and Manley, in Cyprus, both working in countries where single spore vaccines have been very successfully used, compared sapovaccines directly with good spore vaccines, on a small scale. Neither found the results with saponin vaccine good enough to make use of the new method for immunization in the the field. As will be shown, these workers were misled by European publications and thus failed to give saponin a fair trial. The insistance, by Mazzucchi in particular, that saponin vaccine should be made from a virulent strain, and by Hruska, that the safety of sapovaccines depended on the degree of reaction to the saponin, led to the production of vaccines causing such severe local and general reactions that their unsuitability for field use was obvious.

Hruska, Eichhorn and Lyon, Mazzucchi, and others, claimed that cattle, horses, sheep and goats, could all be immunized with the same sapovaccine. Nevertheless, the observations of Staub, Troger, Bory, and Huber, showed that saponin vaccine was not innocuous, and Zofijekski, Megyaszia, and Gerlach, stated that horses should not be inoculated with the cattle vaccine. It is noteworthy that Lederle Bros., in America, now manufacture a separate vaccine for horses, in spite of the early findings of Eichhorn and Lyon. Nevertheless most of the evidence points to a definite, if limited, reduction in virulence by saponin.

A further point, which should be considered when evaluating the results obtained with saponin vaccine, is dosage. Many workers used large doses of spores in the laboratory tests. It was shown, Sterne (1937), Sterne and Robinson (1939), that immunity in anthrax is closely bound up with the number of bacilli in the vaccinating dose. Hall, Hruska, Staub, Hupbauer and Gojkovic, all used fairly massive doses of bacilli, and this must have flattered their results. Several authors have drawn conclusions about the safety of saponin vaccines on the results of laboratory tests. We have shown in a previous paper that such an assumption courts disaster. It cannot be assumed that a vaccine safe for goats kept under laboratory conditions will be at all safe in the field.

This survey of the literature shows, therefore—

(1) that, with few exceptions, workers are convinced that saponin vaccine is better than older vaccines such as Pasteur's or Sobernheim's;

(2) that there is a doubt whether saponin vaccine is superior to a good spore vaccine;

(3) that there is little doubt that saponin reduces, to some extent, the virulence of anthrax strains; but that *virulent* strains cannot be used to prepare vaccines;

(4) that there is no conclusive evidence showing that saponin improves the immunizing power of a strain;

(5) that the new vaccine is not as safe as was first thought.

EXPERIMENTS.

A. Experiments to see whether Saponin Vaccine could be Safely Used under South African Conditions.

[Saponin purissimum or purum (Merck) was used throughout.]

1. *The Effect of Saponin on Virulent Anthrax Strains.*

Table I.

The Effect on Sheep and Goats of Virulent Strains suspended in Saponin according to the Method of Hruska.

Number of sheep.	Dose of virulent spores in 5 per cent. saponin.	Results.
2	2·0 c.c.	† (2), † (3).
2	1·0 c.c.	† (3), † (2).
2	0·5 c.c.	† (2), † (6).
1	0·3 c.c.	L.
1	0·1 c.c.	† (4).
	Dose of virulent spores in 10 per cent. saponin.	
2	2·0 c.c.	† (5), † (7).
2	1·0 c.c.	† (2), † (8).
3	0·5 c.c.	† (5), † (7), L.
1	0·3 c.c.	L.
1	0·1 c.c.	† (6).
Goats.		
1	0·3 c.c. (5 per cent. saponin)	† (8).
1	0·3 c.c. (10 per cent. saponin)	† (8).
Controls.	Virulent spores in distilled water.	
1 sheep	0·3 c.c.	† (6).
1 goat	0·3 c.c.	† (5).

† (2), or † (3) = Died in two or in three days.
L. = Lived.

The results confirmed those of Jezić (1933) and of Huber (1933). Although there was evidence that the saponin modified the action of the virulent spores, we did not consider it worth proceeding with these experiments because the results were too unpromising. It did not appear likely that virulent spores could be incorporated in a vaccine.

A number of experiments were carried out on rabbits and the findings were consistent with those obtained with sheep and goats. Most of the rabbits died, but it seemed that the use of saponin reduced, slightly, the number of deaths.

2. *The Effect on Merino Sheep of a Vaccine strain suspended in five per cent. Saponin.*

Spores of an ordinary vaccine strain were suspended in five per cent. saponin. Sixty sheep were each inoculated with 0·3 c.c., subcutaneously. One lot of twenty were done in the side of the neck, one lot in the inside of the thigh, and another lot under the tail. All sheep were left to run in a paddock.

After twenty-four hours most of the sheep showed extensive oedema and inflammation. A number had large bullae and some showed erosions where the bullae had ruptured. Those done in the leg showed the same local lesions as the others and, in addition, were extremely lame. Some carried or trailed the affected leg. The reactions were still marked after a week.

3. *The Effect on Sheep of Saponin Solution alone.*

Twenty sheep were divided into four groups and each received one c.c. of one of the following solutions: 2·5 per cent. saponin in saline; 2·5 per cent. saponin in 50 per cent. glycerine-saline; 5 per cent. saponin in saline; and 5 per cent. saponin in 50 per cent. glycerine-saline. All the animals were stabled and the inoculations done in the inside of the thigh.

The reactions were severe. Extreme lameness was the rule and open erosions were frequent. The lesions and lameness were still quite marked after 14 days. There were no detectable differences in the lesions and lameness provoked by the saponin in saline, and the saponin in glycerine-saline.

4. *Determination of Suitable Dose of Saponin for South African Conditions.*

Six sheep were each inoculated with a double dose of "Carbozoo" vaccine prepared by the Istituto Sieroterapico Milanese, and six with a double dose of "Carbozoo" from Lederle Laboratories, New York. The reactions were inconsiderable compared with those we got with saponin alone. Thus the violent reactions originally required by Hruska and by Mazzucchi no longer seemed insisted on. We found that 0·5 c.c. of 0·25 to 0·5 per cent.

saponin provoked, in sheep, reactions roughly equivalent to those caused by the imported vaccines. The addition of glycerine (up to 50 per cent.) seemed to have little effect on the character or intensity of the reaction.

5. *Examination of Strains Isolated from "Carbozoo" Vaccines.*

Anthrax strains were isolated from both "Carbozoo" (Milanese) and "Carbozoo" (Lederle). The former, according to the manufacturers, should contain a virulent strain. The latter is stated to contain a Pasteur II strain. The cultures from *both*, however, were typical of vaccine strains and only slightly more virulent than the strains used here, at Onderstepoort, for preparing ordinary single spore vaccine.

A trial batch of spore vaccine—not saponin spore vaccine—was prepared from a strain freshly isolated from "Carbozoo" (Milanese). A test of the virulence of the undiluted glycerine-saline suspension (circa 30 million spores per c.c.) gave the following results:—

2 sheep received.....20·0 c.c. L, L.
2 goats received.....10·0 c.c. L, L.
2 rabbits received ..... 0·1 c.c. L, L.
2 guinea-pigs received..... 0·1 c.c. †(3), †(3).
2 guinea-pigs received..... 0·01 c.c. †(3), †(4).
2 guinea-pigs received..... 0·001 c.c. †(3), †(3).

Sheep which received 0·1 or 0·01 c.c. of this vaccine were solidly immune to a test with a large dose of virulent spores.

Thirty thousand head of cattle were then inoculated with a dose equivalent to 0·01 c.c. of the undiluted suspension (circa 300,000 spores per c.c., and five died. Thus although this strain was a shade more virulent than the one we use, it could, by no stretch of the imagination, be called virulent. According to our criteria [Sterne and Robinson (1939)], batches that kill guinea-pigs as rapidly as this are not issued for large scale inoculations. Trial batches (without saponin), prepared from Lederle Laboratories strain, gave approximately the same results.

Further investigation showed that if cultures from either the Italian or American "Carbozoo" vaccine were allowed to age slightly on agar their virulence decreased. Such strains were then very suitable for preparing ordinary spore vaccines. Their virulence could, if necessary, be easily enchanced by guinea-pig passage. The glycerine-saline spore suspensions prepared from these strains were stable for long periods.

Several batches of spore vaccine (again without saponin) were prepared from strains isolated from the Italian and the American vaccines. These batches were used for inoculating large numbers of cattle. The results of the tests done from 1936 to 1937 are summarized below.

TABLE II.

Summary of laboratory and field tests of vaccine batches prepared from strains isolated from "Carbozoo" vaccines.

Laboratory Tests.					Field Tests.	
No. of merino sheep used.	Strains isolated from.	c.c. Undiluted vaccine administered.	Result.	Result of test (three weeks later) with large dose virulent spores.	No. of doses issued to field, 1936–37.	Results.
12	"Carbozoo" (Milanese)	20·0 c.c.	Lived	All lived	3 Million	Excellent. Safe, and immunized well.
20		0·1 c.c.	Lived	4 died		
31		0·01 c.c.	Lived	6 died		
4	Carbozoo (Lederle)	20·0 c.c.	1 died	All lived	½ Million	Immunized well, but sometimes dangerous to well-bred cattle.
8		0·1 c.c.	Lived	All lived		
8		0·01 c.c.	Lived	All lived		
Controls.				With small dose Virulent Spores.		
36	—	—	—	All died		

All these lots of vaccine were issued at dilutions varying from 1/25 to 1/100 of the concentrated suspension (1,000,000 to 250,000 spores per c.c.). The results showed that, whether saponin did or did not have the effect claimed for it, the strains isolated from saponin vaccines gave excellent results, without saponin. These strains were as effective as the Boshoff strain which we have used successfully for so many years.

Discussion of Preliminary Experiments.

(1) Virulent strains suspended in saponin could not be used as vaccines.

(2) The amounts of saponin recommended by Mazzucchi and by Hruska gave reactions that were too severe for South African conditions. In this country, where myiasis is not uncommon, open lesions cannot be tolerated. The extreme lameness, and consequent impairing the animal's foraging ability, is also a serious drawback under extensive farming conditions.

(3) Anthrax strains isolated from "Carbozoo" (Milanese) and "Carbozoo" (Lederle) were typical vaccine strains. They could be used very satisfactorily without saponin.

(4) A dose of 0·5 c.c. of 0·5 per cent. saponin solution produced, in sheep, reactions roughly equivalent to those evoked by the imported vaccines. This dose, 0·5 c.c., was chosen because, for technical reasons, it was desirable to keep the small animal dose at 0·5 c.c. and the large animal dose at 1·0 c.c.

B. Experiments to see Whether Saponin would (1) Modify the Virulence of Vaccine Strains and (2) Improve the Immunity Elicited by such Strains.

The preliminary experiments showed that "Carbozoo" vaccine contained an attenuated strain in probably not more than 2·0 per cent. saponin. We wished to know whether saponin, in doses which were tolerable under our conditions, would fulfil the claims made for it and justify the expense and inconvenience (foaming) of using this substance extensively. We were loth to give up the convenience of using glycerine in the vaccine so the effect of this was also investigated.

1. *Experiments on Guinea-pigs to see whether Saponin could Reduce the Virulence of Vaccine Strains.*

Mazzucchi and others claimed that saponin reduced the virulence of anthrax strains without impairing their ability to immunize and that even virulent strains could be used as vaccines, if suspended in saponin. This latter contention is unlikely in view of the results obtained by us and by other workers. It was decided therefore to try the effect of saponin on attenuated strains.

The tests had necessarily to be carried out on small animals, because sheep and larger animals are, in the laboratory, perfectly resistant to vaccine strains with, or without, saponin. The amount of saponin in each dose of vaccine given to the guinea-pigs was

chosen so that the resulting reactions should be comparable with those desired in large animals. It was felt that doses causing extensive necrosis would not give the information required; that is, information as to the possible effect of saponin on vaccine strains, in practice.

Guinea pigs were inoculated with 0·1 c.c. of washed spores of a vaccine strain suspended in (1) saline; (2) 1 per cent. saponin-saline; (3) 50 per cent. glycerine-saline; (4) 1 per cent. saponin in 50 per cent. glycerine-saline.

The spores were left in saponin for 20 days. The dose of vaccine was chosen so that not all the guinea-pigs would die. Thus there was a greater possibility of differences between the groups being shown. Preliminary experiments had already shown that these differences would *not* be large. The results are summarized in Table III.

Analysis of Results shown in Table III.

(*a*) *Groups* 1, 2, 3, 4.—The numbers of guinea pigs that died at twelve-hourly intervals were compared by Fisher's X^2 method. It was found that there was a significant difference ($P<0·01$) between the rates at which the guinea-pigs died in the different groups.

The numbers of survivors in the four groups did not differ significantly (P circa 0·1).

(*b*) *Groups* 1 *and* 3 (*saline and glycerine-saline*).—The number of deaths was compared with the number of survivors at each twelve-hour period. There was a significant difference between the two groups up to 156 hours.

The total number of deaths and survivors (240 hours) also differed significantly in the two groups ($P<0·02$).

(*c*) *Groups* 1 *and* 3 *compared with* 2 *and* 4 (*groups without saponin compared with groups with saponin*).—There was a highly significant difference in the number of deaths up to 108 hours, and thereafter the difference disappeared.

(*d*) *Group* 1 *compared with group* 2 (*saline versus saline-saponin*).—There was no significant difference up to 60 hours, but thereafter the groups differed, significantly up to 108 hours. After that, again no difference.

(*e*) *Group* 3 *compared with group* 4 (*glycerine-saline versus glycerine-saline-saponin*).—Again, there was a significant difference between the groups up to 108 hours.

(*f*) *Comparison of effect of saponin on saline and glycerine respectively.*—There was a significant reaction, up to 96 hours, between glycerine and saline with saponin and no saponin. After 96 hours this reaction was no longer evident.

The following conclusions can be drawn from this analysis:

From (*b*).—Spores suspended in glycerine were appreciably more virulent than those suspended in saline. There was not only a more rapid initial death rate, but also significantly fewer survivors in the glycerine group.

Table III.

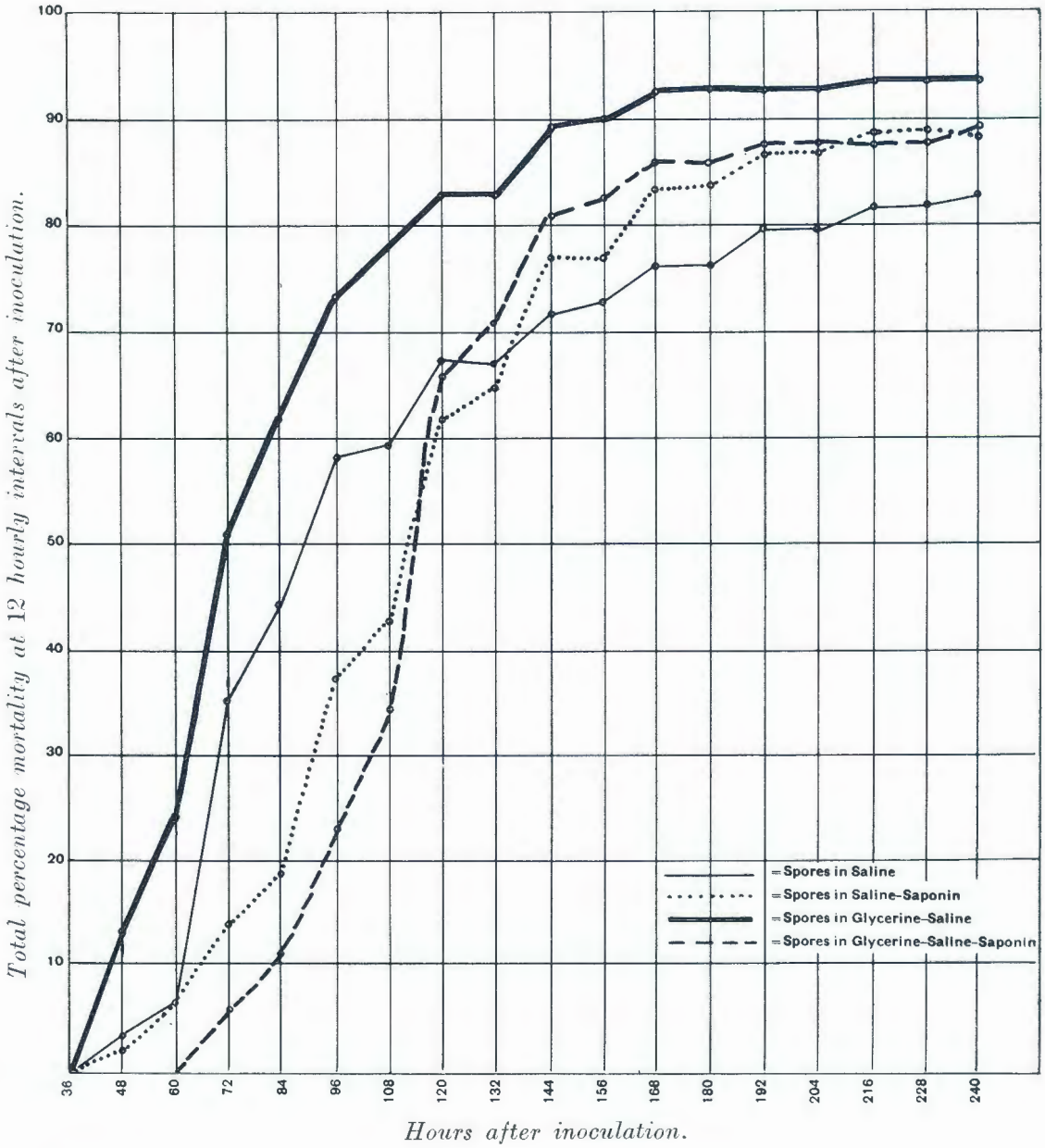
The effect on guinea-pigs of attenuated spores suspended in (1) saline; (2) saponin-saline; (3) glycerine-saline; (4) saponin-glycerine-saline.

Hours after Inoculation.	1. Saline. No. of Guinea-pigs.			2. 1 Per Cent. Saponin-saline. No. of Guinea-pigs.			3. 50 Per Cent. Glycerine-saline. No. of Guinea-pigs.			4. 1 Per Cent. Saponin in 50 Per Cent. Glycerine-saline. No. of Guinea-pigs.		
	Dead.	Alive.	Total.	Dead.	Alive.	Total.	Dead.	Alive.	Total.	Dead.	Alive.	Total.
48	2 (2·3)		87	2 (3·5)		58	15 (13·5)		111	0 (0)		60
60	6 (6·9)		87	4 (6·9)		58	27 (24·3)		111	0 (0)		60
72	31 (35·6)		87	8 (13·8)		58	57 (51·4)		111	4 (6·7)		60
84	39 (44·8)		87	11 (19·0)		58	69 (62·2)		111	7 (11·7)		60
96	51 (58·6)		87	22 (37·9)		58	82 (73·9)		111	14 (23·3)		60
108	52 (59·8)		87	25 (43·1)		58	85 (76·6)		111	21 (35·0)		60
120	59 (67·8)		87	36 (62·1)		58	93 (83·8)		111	40 (66·7)		60
132	59 (67·8)		87	38 (65·5)		58	93 (83·8)		111	43 (71·7)		60
144	63 (72·4)		87	45 (77·6)		58	100 (90·1)		111	49 (81·7)		60
156	64 (73·6)		87	45 (77·6)		58	101 (91·0)		111	50 (83·3)		60
160	67 (77·0)		87	49 (84·5)		58	104 (93·7)		111	52 (86·7)		60
180	67 (77·0)		87	49 (84·5)		58	104 (93·7)		111	52 (86·7)		60
192	70 (80·5)		87	51 (87·9)		58	104 (93·7)		111	53 (88·3)		60
204	70 (80·5)		87	51 (87·9)		58	104 (93·7)		111	53 (88·3)		60
216	72 (82·8)		87	52 (89·7)		58	105 (94·6)		111	53 (88·3)		60
228	72 (82·8)		87	52 (89·7)		58	105 (94·6)		111	53 (88·3)		60
240	73 (83·9)	14 (16·1)	87 (100)	52 (89·7)	6 (10·3)	58 (100)	105 (94·6)	6 (5·4)	111 (100)	54 (90·0)	6 (10)	60 (100)

The first columns represent the actual number of guinea-pigs; the figures in brackets represent percentages.
Those guinea-pigs that were alive at 240 hours, remained alive.

FIGURE I.

The mortality rates in four groups of guinea-pigs inoculated with attenuated spores suspended in different menstrua.



From (*c*), (*d*), (*e*).—The addition of saponin reduced the virulence of both glycerine and saline spore suspensions. This was shown only in a lag in the rate of dying in the saponin groups; for eventually there was no significant difference in the number of survivors.

From (*f*).—There was practically no difference in mortality in the saline-saponin and glycerine-saline-saponin groups. The reaction demonstrated in (*f*) shows that saponin reduced the virulence of glycerine suspensions more than it reduced the virulence of saline suspensions.

These conclusions are clearly demonstrated in Figure 1.

2. *Experiments on Sheep to see whether Saponin Increased the Immunity Produced by Vaccine Strains.*

It has already been stated that only the grossest differences in virulence of vaccine strains can be detected in large animals kept under laboratory conditions. The experiments noted below, therefore, were designed only to see whether spores in saponin produced a better immunity than similar spores without saponin.

Below are summarized the results of several experiments where the immunity produced by spores of vaccine strains suspended in glycerine-saline was compared with the immunity elicited by the same number of spores in glycerine-saline plus saponin (1—2·5 per cent.), or in saline plus saponin. All the sheep were tested with a large dose of virulent spores three weeks after inoculation, while control sheep received one-tenth to one-thousandth of this dose.

TABLE IV.

Comparison of immunity produced in sheep by spores with, and spores without saponin.

No. of sheep.	Inoculated with.	Tested three weeks later with.	Result of test with virulent spores.			
			Lived.	Died.	Mean No. of days lived by sheep that died.	Range of days lived by sheep that died.
64	Vaccine spores suspended in saponin	Large dose virulent spores	53 (83%)	11 (17%)	9·6	3 – 30
32	Vaccine spores without saponin	Large dose virulent spores	19 (59%)	13 (41%)	5·7	2 – 22
22	Nil (controls)	Small dose virulent spores	0 (0%)	22 (100%)	2·9	2 – 5

There were significantly more survivors ($P<0{\cdot}02$) in the sheep immunized with spores in saponin.

The mean survival periods (in days) of the sheep which died in the three groups were compared by an analysis of variance technique. It was found that the sheep that died in the saponin group lived significantly longer than the controls. No other significant differences were found; but it should be noted that the controls would have died still sooner had they received the same dose of virulent spores as the immunized sheep.

The following table summarizes a small experiment done to see whether there was any difference between the immunity produced by saponin-suspensions with or without glycerine.

TABLE V.

The effect of glycerine on the immunity produced by spores suspended in saponin.

No. of sheep.	Inoculated with.	Tested three weeks later with.	Result of test with virulent spores.	
			Lived.	Died.
16	Vaccine spores in glycerine-saline-saponin	Large dose virulent spores	13	3
16	Vaccine spores in saline-saponin	Large dose virulent spores	14	2
4	Nil (controls)	Small dose virulent spores	0	4

This test therefore showed no difference in behaviour between spores suspended in saponin-glycerine-saline or saponin-saline.

A further test was done to show the effect of saponin on vaccine spores. An *avirulent* spore suspension, prepared from an uncapsulated variant of a virulent strain (Sterne, 1937), was used. This suspension, now eight months old, had been used as an ordinary vaccine, but was now about to be discarded owing to age. Equal amounts of the spore suspension (at the dilution used for routine issue) were suspended in glycerine-saline and in glycerine-saponin-saline. Sheep were inoculated as shown in Table VI.

We deliberately used an aged and deteriorated vaccine. Three weeks later, the sheep were tested with a very large dose of virulent spores. We thought that if a fair number of the sheep were killed by the test, differences between the groups would be more likely to show.

TABLE VI.

The effect of saponin on the immunity produced by an avirulent spore vaccine.

No. of sheep.	Inoculated with.	Tested three weeks later with.	Results of test with virulent spores	
			Lived.	Died.
15	Avirulent spores in 1 per cent. saponin–50 per cent. glycerine-saline	Large dose virulent spores	13	2
15	Avirulent spores in 50 per cent. glycerine-saline	Large dose virulent spores	3	12
2	Nil (controls)	Small dose virulent spores	0	2

This result is highly significant ($P<0{\cdot}01$) and is an additional proof of the efficacy of saponin in improving the immunity produced by vaccines. It is interesting to see that this effect is also shown with an avirulent uncapsulated strain.

Conclusions.

(1) The immunizing power of vaccine strains was considerably increased by suspending them in saponin. This increase was also evident in the case of an avirulent uncapsulated strain.

(2) No difference was detected in the behaviour of saponin with or without glycerine.

C. FIELD TESTS WITH SAPONIN VACCINES.

1. *Large Scale Immunization with Vaccine Strains Suspended in Glycerine-saline, with and without saponin.*

The vaccines used were suspensions of spores made from strains isolated from " Carbozoo " (Milanese). They were so attenuated that they could safely be used without saponin. The spores were diluted for issue (*a*) in 50 per cent. glycerine-saline, (*b*) in 50 per cent. glycerine-saline plus 0·5 per cent. saponin. Preliminary field work showed that vaccine prepared in this way was well tolerated, and did not cause undesirable local reactions.

A large scale test was then carried out as follows: In the Transkei, in 1937, 598,250 cattle were inoculated with saponin spore vaccine, and 951,720 cattle with the same lots of spore vaccine, without saponin. All the cattle (1,549,970) were inoculated within two months (May-June). The results are summarized below.

The Transkei—a homogeneous administrative area—was chosen for the tests because it was possible to inoculate *all* the cattle in the area. Here there exists a rigid control and supervision by a large veterinary staff. This control is aimed chiefly at eradicating East Coast fever, but advantage has been taken of it to test the feasibility of controlling anthrax by inoculation alone. Full details of the method of counting, checking and controlling the animal population will be given in another paper dealing with the control of anthrax by immunization. Now it must suffice to say that the control is real and extraordinarily effective.

TABLE VII.

Field tests with spore vaccines with and without saponin.

No. of Cattle Inoculated.	Inoculated with.	Date of inoculation.	No. of deaths, June, 1937 to May, 1938.	Deaths Percentage.
598,250	Saponin-spore vaccine	May and June, 1937	34	·006
951,720	Same vaccine without saponin	May and June, 1937	40	·004

The data give no indication that one vaccine immunized better than the other. There were fewer deaths in the non-saponin lot but the proportions are without significance. No figures are available for uninoculated cattle. However, the incidence of anthrax in areas bordering on the Transkei, where inoculation is more haphazard, is very much higher. These figures, too, will be given in another paper.

2. *Safety of Sapo-vaccines in the Field.*

It may be argued that saponin vaccines would yield better results in the field if prepared from more virulent strains. The following observations throw light on this point:—A batch of ordinary spore vaccine, which had passed all the laboratory tests satisfactorily, was sent to the field for testing. In the first test, on 300 head of cattle, one died and several showed swellings. For this reason the batch was withdrawn. We thought, however, that it would provide a satisfactory, and a not too severe test of the effect of saponin in reducing virulence. Accordingly, further issues of this batch were made suspended now in 0·5 per cent. saponin. (Dose 1·0 c.c.—as was shown earlier, larger concentrations of saponin produced too severe reactions).

The results, on the whole, were very good, but there were some complaints of deaths following inoculation. Two such complaints we were able to investigate. The following table summarizes the findings.

TABLE VIII.

Deaths following inoculation of saponin vaccine.

No. of Sheep Inoculated.	Deaths following Inoculations.	Bacteriological Findings.
900	†(5), †(5) †(6), †(7) †(8), †(9) †(8), †(9)	All these sheep showed anthrax septicaemia. The bacilli isolated on culture were typical of vaccine strain inoculated.
	†(5)	Dead in 5 days.

Thus although the spores had been in contact with the saponin for a considerable time, we had incontrovertible evidence of deaths caused directly by saponin vaccine.

Some other attempts were made to issue stronger vaccines incorporated in saponin, but these vaccines now and then gave rise to alarming reactions, and eventually we were compelled to give up trying to use stronger strains and to employ—for preparing saponin vaccine—strains that could be safely issued without saponin.

Nevertheless saponin did, to a moderate extent, reduce virulence. We are convinced, from experience, that there would have been more trouble with some of the stronger vaccines had saponin been omitted. Of the several million cattle inoculated with saponin vaccine, more than a million have been done intramuscularly. The reactions have not been more severe than those found in the animals inoculated subcutaneously. The severe reactions and deaths encountered by Rivadelo (1937), in the Philippines, which he ascribed to accidental intramuscular inoculation, were probably the result of using too potent a vaccine. We have some evidence, however, that intramuscular inoculation increases the chances of getting a gas-gangrene infection.

DISCUSSION.

Both Hruska and Mazzucchi said that virulent strains suspended in saponin could be used to immunize animals against anthrax. Mazzucchi, indeed, claimed this as one of the principal advantages of using saponin. We, however, found that the reduction in virulence produced by saponin was, for practical purposes, negligible, and that saponin-treated virulent strains still killed sheep and goats readily. We also, like others, failed to isolate a virulent strain from "Carbozoo" vaccine.

The amounts of saponin originally advocated by Hruska, and by Mazzucchi, caused severe reactions. Whatever the merits of such reactions might be, they cannot be tolerated in large-scale inoculations. Accordingly, we lowered the saponin content of our preparations until the reactions they produced were compatible with their

use under South African conditions. All subsequent experiments were conducted on this basis. Other workers, as has been mentioned, have also reduced considerably the amounts of saponin originally prescribed.

Extensive experimentation on guinea-pigs showed that saponin reduced the virulence of vaccine strains, slightly. This occurred whether glycerine was present in the suspension or not. The reduction in virulence, however, was much less than that found by Hruska, and Mazzucchi, and was only shown by a retarding of the death-rate. Field experiments confirmed the laboratory results, for moderately strong spore vaccines, even with saponin added, proved unsafe in practice. Deaths occurred in sheep when saponin vaccines made with spores only slightly more virulent than ordinary vaccine spores were used. We feel, therefore, that the results of the guinea-pig experiments truly reflect the reduction in virulence caused by saponin in the concentrations used here.

These experiments also showed that glycerine enhanced the virulence of vaccine strains. A similar observation was made by Viljoen and Scheuber (1927) on *Cl. chauvoei*. The level of virulence of anthrax strains after the addition of saponin was the same, whether glycerine was present or not.

We compared the immunity produced by spore vaccines with and without saponin, in a large field-test on about one-and-a-half million cattle, and found no difference. Ordinary spore vaccine protects so well that no difference was to be expected in a test of this sort.

Laboratory experiments on sheep showed conclusively that saponin improved immunity. This enhancement also occurred with an uncapsulated, avirulent variant. A striking observation, which is opposed to the findings of Staub, Ramon and Staub, and Jezić, was that a poor vaccine suspended in saponin produced excellent immunity.

We thought, before the completion of these experiments, that the success of saponin vaccines in Europe and America was due mainly to the incorporation, in these preparations, of an excellent immunizing strain. This probably explains, partly, why sapo-vaccines have not been startling successes in countries like Cyprus, the Dutch East Indies, and South Africa, where good types of spore vaccine have long been used. Nevertheless, our laboratory results substantiate, unequivocally, Mazzucchi's claims that saponin increases the immunity produced by anthrax vaccines.

Since the completion of these experiments we have prepared and issued large amounts of saponin vaccine. (Spores suspended in 0·5 per cent. saponin in 50 per cent. glycerine-saline: dose 1·0 c.c. for cattle). The strains incorporated recently have been, if anything, milder than those used in plain spore vaccine, because the action of saponin in enhancing immunity appears to be more important than its action in reducing virulence. Thus by using saponin, and by using weaker strains, the safety of the vaccine has been improved. It is still necessary to issue separate vaccines for goats and horses. The field results with avirulent strains will be published in another paper.

SUMMARY AND CONCLUSIONS.

(1) Virulent anthrax strains incorporated in saponin retained much of their virulence, and could not be used in vaccines.

(2) Italian and American commercial saponin vaccines examined by us did not contain virulent strains.

(3) Not more than 0·5 to 1·0 per cent., saponin should be used, where the dose for bovines is 1·0 c.c. and for sheep 0·5 c.c.

(4) Saponin reduced slightly the virulence of vaccine strains for guinea-pigs.

(5) Glycerine enhanced the virulence of vaccine strains for guinea-pigs.

(6) A large-scale field test failed to show any difference between saponin vaccine and ordinary spore vaccine.

(7) Laboratory tests, on sheep, showed that saponin considerably increased the immunizing power of ordinary vaccine strains. This phenemenon could also be demonstrated in the case of an avirulent, uncapsulated variant.

(8) It is suggested that saponin should be used with mild strains to improve immunity, rather than with strong strains to reduce virulence.

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