ON THE SERO-DIAGNOSIS OF GLANDERS

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Notwithstanding the discovery of the glanders bacillus the diagnosis of chronic glanders has always been difficult. Later, mallein came into use, but since the results obtained therewith did not realize all expectations, new sero-logical methods were introduced.

Of late two methods were especially used with success, the complement deviation and the agglutination. In the course of this paper these two methods will be described. Another method, the precipitine test, which I also tried, gave unsatisfactory results. The subcutaneous injection of mallein did not prove to be a complete success. Several observations were made at the Laboratory, where, with mallein, a reaction was noted in horses which were not suffering from glanders. I know of some instances where the mallein reaction, both temperature and local, was quite typical, and when the animal was subsequently killed no lesions of glanders were found. On the other hand I saw cases where an animal passed the mallein test with negative results, and yet was affected with glanders. I do not think it necessary to enter into the results of such incidents, but they were the reason for undertaking the experiments given in this paper.

1.—Complement Deviation Test.

This method was first described by E. de Haan. At the beginning of our experiments we used the method as explained in his paper; afterwards for special scientific investigations I adopted *Wassermann's* method. Later *Schütz* and *Schubert* carried out experiments concerning the use of the complement deviation test as a practical sero-logical method for the diagnosis of glanders, and they recognized the value of the reaction and its importance in the diagnosis of this disease. Recently *Miesner* and *Trapp* compared the complement test with the same reaction in syphilis. I do not think it necessary to note the details of this publication here; we shall compare them later with our results.

I will give a short description of the technique used by me, this being more or less the same as that described by *Schütz* and *Schubert*. All the components of the test were prepared in the manner described in the later part of this paper, and were also successfully used in other complement deviation experiments. The tests were always carried out in two stages. The first examination is called the preliminary or orientation test, the second the complementary test. They were carried out as follows: All sera which in the quantity of 0·2 or 0·1 c.c. gave a specific deviation were tested in the second test in descendent doses of 0·1, 0·05, 0·02, 0·01 c.c., and all the sera which gave a deviation with 0·02 c.c. had to pass the agglutination test. By this means about 997 sera were examined, belonging to 387 horses, 237 mules, and 25 donkeys. The animals whose serum gave a positive reaction were killed, and the *post-mortem* examination supported the result of the reaction. In a few cases the method was also used successfully for the differentiation of doubtful lung nodules. In the following tabulated statement a test is described:—

No. 1.—*Orientation Test.*

Equines Number.	Immune Serum.	Complement. (Generally 0·03.)	Antigen. Glanders Bacillus Extract. 1:100.	Haemolytic Amboceptor. (Titre gen. 1:1200.)	Sheep or Cattle Blood Corpuscles. 5 %	Physiological Water. 0·85%	Result.
Horse 3781	0·2	1·0	1·0	1·0	1·0	1·0	Haemolysis.
	0·2	1·0	—	1·0	1·0	2·0	,,
Horse 4447	0·2	1·0	1·0	1·0	1·0	1·0	Very slight deviation.
	0·2	1·0	—	1·0	1·0	2·0	,, ,,
Mule 4774	0·1	1·0	1·0	1·0	1·0	1·0	Haemolysis.
	0·1	1·0	—	1·0	1·0	2·0	,,
Mule 4776	0·1	1·0	1·0	1·0	1·0	1·0	Slight deviation.
	0·1	1·0	—	1·0	1·0	2·0	,, ,,
Horse 3253	0·2	1·0	1·0	1·0	1·0	1·0	Complete deviation.
	0·2	1·0	—	1·0	1·0	2·0	Haemolysis.
Mule 4138	0·1	1·0	1·0	1·0	1·0	1·0	Complete deviation.
	0·1	1·0	—	1·0	1·0	2·0	Haemolysis.
Mule 4809	0·1	1·0	1·0	1·0	1·0	1·0	Complete deviation.
	0·1	1·0	—	1·0	1·0	2·0	Haemolysis.
Mule 4537 Both	0·2	1·0	1·0	1·0	1·0	1·0	Deviation.
	0·2	1·0	—	1·0	1·0	2·0	,,
Horse 4604	0·2	1·0	1·0	1·0	1·0	1·0	Haemolysis.
	0·2	1·0	—	1·0	1·0	2·0	,,
Horse 4625	0·2	1·0	1·0	1·0	1·0	1·0	,,
	0·2	1·0	—	1·0	1·0	2·0	,,
Horse 4806	0·2	1·0	1·0	1·0	1·0	1·0	,,
	0·2	1·0	—	1·0	1·0	2·0	,,

Test-tube No.	Immune Serum.	Antigen.	Complement.	Haemolytic Amboceptor.	Blood Corpuscles. 5 %	Physiological Water.	Result.
I.	Generally Serum of Horse 4481. —	1·0 (0·001)	1·0	1·0	1·0	2·0	Haemolysis.
II.	—	2·0 (0·002)	1·0	1·0	1·0	1·0	,,
III.	0·02	—	1·0	1·0	1·0	2·0	,,
IV.	0·4	—	1·0	1·0	1·0	2·0	,,
V.	0·2	—	1·0	—	1·0	3·0	Complete deviation.
VI.	0·2	—	—	1·0	1·0	3·0	,, ,,
VII.	—	—	1·0	1·0	1·0	3·0	Haemolysis.
VIII.	—	—	1·0	—	1·0	3·0	Complete deviation.
IX.	—	—	—	—	1·0	3·0	,, ,,
X.	—	—	—	—	1·0	4·0	,, ,,

Epicrisis.—From these experiments, where the controls were always carried out with serum of a horse suffering from glanders, we can state that the mules 4138, 4809, and horse 3253 showed specific deviations of the complement. Mule 4537, with 0·1 serum (it was heated at a temperature of 62° C.) showed non-specific deviation. All these four animals had to pass the complementary test and at the same time the agglutination test. The results of the first test are noted for two cases in the following tabulated statement :—

TABLE 1.

MULE 4138.

Immune Serum.	Complement. (Generally 0·03).	Antigen 1 : 100.	Haemolytic Ambo-ceptor.	5 % Washed Blood Corpuscles.	Physiological Water. 0·85 %	Result.
0·2	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·2	1·0	—	1·0	1·0	2·0	Slight deviation.
0·15	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·15	1·0	—	1·0	1·0	2·0	Haemolysis.
0·1	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·1	1·0	—	1·0	1·0	2·0	Haemolysis.
0·05	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·05	1·0	—	1·0	1·0	2·0	Haemolysis.
0·04	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·04	1·0	—	1·0	1·0	2·0	Haemolysis.
0·03	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·03	1·0	—	1·0	1·0	2·0	Haemolysis.
0·02	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·02	1·0	—	1·0	1·0	2·0	Haemolysis.
0·01	1·0	1·0	1·0	1·0	1·0	,,
0·01	1·0	—	1·0	1·0	2·0	,,
0·005	1·0	1·0	1·0	1·0	1·0	,,
0·005	1·0	—	1·0	1·0	2·0	,,

Controls.

0·01	1·0	—	1·0	1·0	2·0	Haemolysis.
0·4	1·0	—	1·0	1·0	2·0	Slight deviation.
0·2	1·0	—	—	1·0	3·0	Complete deviation.
0·2	—	—	1·0	1·0	3·0	,, ,,

TABLE II.

HORSE 3253.

Immune Serum.	Complement.	Antigen.	Haemolytic Amboceptor.	Blood Corpuscles. 5 %	Physiological Water.	Result.
0·2	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·2	1·0	—	1·0	1·0	2·0	Haemolysis.
0·1	1·0	1·0	1·0	1·0	1·0	Complete deviation.
0·1	1·0	—	1·0	1·0	2·0	Haemolysis.
0·05	1·0	1·0	1·0	1·0	1·0	Slight deviation.
0·05	1·0	—	1·0	1·0	2·0	Haemolysis.
0·02	1·0	1·0	1·0	1·0	1·0	,,
0·02	1·0	—	1·0	1·0	2·0	,,
0·01	1·0	1·0	1·0	1·0	1·0	,,
0·01	1·0	—	1·0	1·0	2·0	,,

Controls.

0·02	1·0	—	1·0	1·0	2·0	Haemolysis.
0·4	1·0	—	1·0	1·0	2·0	,,
0·2	1·0	—	—	1·0	3·0	Complete deviation.
0·2	—	—	1·0	1·0	3·0	,, ,,

The doses of serum used, as shown in Table I, revealed the exact titre, but for routine the Table II used by *Schütz* and *Schubert* is quite sufficient. The results obtained this way are the following :—

No deviation of the complement .. { Equidae free from glanders 582
Equidae with glanders .. 2

Titre .. .. .. (Binding coefficient) ("Bindungswert") ..

0·3	: 2	Mules.
0·2	: 8	
0·1	: 36	(mallein and piroplasmosis).
0·05	: 5	
0·02	: 18	
0·01	: 4	

Out of 649 equines suspected for glanders 71 animals gave deviations with glanders bacilli extract, indicating that they were suspected to be suffering from the disease, but the agglutination test, and in several cases a second complement deviation test four weeks later, showed that only 27 had to be considered to be infected with glanders. With the complement deviation and agglutination tests 25 out of 27 suspected horses and mules were proved to be infected with the disease. The *post-mortem*

examination supported the diagnosis. Two doubtful reactions with 0·3 c.c. serum and an agglutination in a dilution of 1 : 500 refer to two mules which became infected with the disease by infusion with blood from a mule suffering from chronic glanders. The serum was collected on the seventh day after infusion, at a date when other animals treated in a similar way showed progressive symptoms of glanders, which the two mules had not yet clinically developed on the date the serum was taken. Of the healthy animals the serum of forty-eight showed deviations similar to those observed in horses affected with glanders, but only two of them come into consideration, as of the other forty-six some had been treated with mallein a short time previously, and the remainder were affected with piroplasmosis. The serum of these two doubtful horses showed the titre of 0·05. The sera came from animals which had been hyperimmunized against horse-sickness, and bled several times, and it may be that the anaemia produced had a certain influence on the complement deviation substance of the sera. I think it would be advisable to carry out further experiments in this direction. The results obtained are good, and inaccuracies are but few, so that with sufficient controls, and with a second test, or by means of the agglutination test, the complement deviation is at present the most valuable method for recognizing glanders in equidae. By this method I think we are enabled to detect glanders with more certainty than with mallein. *So far not one glandered animal has passed our test without having been recognized to be infected with the disease, and especially in all those cases where the mallein reaction gave no definite result the complement deviation demonstrated the nature of the disease at once.*

General Method of the Complement Test.

The following components are necessary :—

1. The immune serum (glanders serum).
2. The antigen (extract of glanders bacillus).
3. Complement (guinea pig serum).
4. Haemolytic serum for the red blood corpuscles used.
5. An emulsion of red blood corpuscles (sheep or cattle).

Into test tubes 1 or 2 c.c. of normal saline solution are added to 0·1 c.c. immune serum ; then in the first tube 1 c.c. antigen (1 : 100) is added, and the second tube (without antigen) will be used as a control. The complement is added to both tubes in a solution which is found by the orientation test. The contents of the tubes are slightly shaken and then incubated at 37° C. for about an hour. Afterwards, to each tube, the inactivated haemolytic system is added, and the tubes are incubated again for two hours at 37° C. They are then kept at room temperature, or in the summer in a cold room, and the result is noted twelve hours later. The components for the test will be referred to under special headings.

A.—The Immune Serum.

By the term immune serum we understand the serum which contains the specific antibodies (glanders serum). The antibodies in glanders have a tendency to fix their specific antigen. That this really takes place is shown after the addition of the haemolytic system, when no dissolution of the red blood corpuscles is observed. A few words on the collection of the serum may be of a certain interest.

1. *The collection of blood and preservation of the serum.*—The blood is generally collected by means of a sterile canula from the jugular vein, and

30 c.c. are placed in sterilized test-tubes. The clear serum is collected by means of a pipette, and hermetically closed in small brown bottles. Experience here has shown that serum kept without any preservative in the ice-chest gave the same reaction at the end of a year as at the beginning. Care has to be taken to keep out light, because sunlight inactivates a serum in six to eight hours. These observations correspond with those of *Miessner* and *Trapp*. Slight decomposition of the serum has no influence; in advanced stages a serum which does not contain antibodies may give non-specific reactions. In field work the high temperatures favour the decomposition of badly collected sera, and therefore we added a small quantity of 5 per cent. carbolic acid to the brown bottles. Bottles sterilized and prepared in this manner with a capacity of 20 c.c., and containing 0·05 carbolic acid, are sent to the Government Veterinary Surgeons by the Institute, if requested. Formalin is useless, but a crystal of thymol can also be used. Several times we found that serum collected from animals suffering from piroplasmosis became slightly coagulated when mixed with carbolic acid. For scientific experiments we preserved all our serum without any additions.

2. *Uninactivated and inactivated sera.*—For the complement deviation test only inactivated sera, viz., sera which have been heated at 56° C. for half an hour, were used. The complement was naturally destroyed by this process. In a test a control was generally made with such serum (for this see my Table No. 6). *Schütz* and *Schubert* and *Miessner* and *Trapp* give a few more details on the importance of using such inactivated sera, because it is a well-known fact that a certain proportion of equine sera contain non-specific bodies, which deviate the complement without any extract being added. It may be mentioned that although under certain conditions uninactivated sera may give good results we always found it necessary to inactivate our sera.

To demonstrate the presence or absence of non-specific bodies, two test-tubes, of which one contained the extract as shown above, were used in every test. A test could only then be called perfect if the control tubes showed haemolysis. How long and at what temperature a serum had to be heated in order to be inactivated was found by the examination of a large number of mule sera. *Miessner* and *Trapp* found that a temperature of 56° C. is not sufficient for destroying all the non-specific bodies in the serum of a mule, and I had a good opportunity to confirm this observation, as the spontaneous deviation in mule sera was often noticed in the dose of 0·05 c.c. Several experiments showed that the sensibility to heat of these non-specific fixing substances did not in all instances correspond with the observation of *Miessner* and *Trapp*; about 20 per cent. of the mules which I used still showed a deviation at a temperature of 60° C. Accordingly I heated all the sera to the maximum possible, that is 62°-63° C., and after this I could use another 15 per cent. for the complement deviation test. The other 5 per cent. were not affected by these temperatures, and only the agglutination test helped us out of these difficulties. I could not determine the nature of the non-specific deviation, and the circumstances under which they acted, and it would be advisable to undertake further experiments to elucidate these points. Heating during twenty-five to thirty minutes at 60° to 63° C., as was shown by experiment, did not have any influence on the action of the specific glanders antibodies of equidae. A temperature of 65° C. destroyed all these bodies, and these facts correspond with the statements of *Miessner* and *Trapp*.

Spontaneous coagulation at 60° C. takes place very rarely. From a practical point of view it is, therefore, advisable for all orientation or preliminary experiments to heat the sera of equidae in a water bath at 60° C. during thirty minutes.

3. *Dosing of the sera.*—Descendent doses from 1·0 to 0·005 c.c. were generally used for exhaustive investigations. As *Schütz* and *Schubert* stated, doses from 0·2 to 0·1 c.c. are quite sufficient to demonstrate a reaction, and sera from 0·2 c.c. downwards generally gave a specific reaction, if they contained the antibodies. The sera of healthy equidae deviated very rarely in doses from 2·0 c.c. downwards to 0·01 c.c., but there were mules which deviated in the dose of 0·2 c.c. We therefore always used the doses from 0·1 c.c. of mule serum, and never failed to obtain a reaction if the animals really had glanders.

4. *The immune bodies in the serum.*—The number of glanderous animals examined by myself was not large, and therefore I was not in the position to make exhaustive investigations into the specific glanders bodies. *Miessner* and *Trapp* showed that the complement deviation coefficient (Bindungswert, *Miessner* and *Trapp*) increases from the sixth until the seventh day after the infection, and after a certain time falls continuously to a normal limit. I was able to make confirmatory observations in this matter on three mules which all became infected at the same date and under the same circumstances. The specific bodies were observed to be present in one mule on the seventh day. In the two other mules of the same lot they were only seen on the tenth day. The increase of the immune bodies was not a rapid one, as *Miessner* and *Trapp* recorded, the coefficient changing from 0·1 to 0·02. On the contrary the agglutination was rather high (1–1000 and 1–2000); therefore it seems that the formation of the fixing immune bodies takes place slowly after the formation of the agglutining substances. Accordingly, in cases of recent infection, the agglutination test appears to be preferable. Based on other observations with Johannesburg horses we could not confirm the fact that the immune substances do not disappear as rapidly as was stated by *Miessner* and *Trapp*. Four horses which belonged to a batch amongst which an outbreak of glanders had occurred, and which gave positive results to the mallein test, were kept at the Institute and still showed specific antibodies in the dose of 0·1 and 0·05 c.c. after a year had elapsed since the first test. The *post-mortem* examination of these horses demonstrated in two cases chronic glanders lesions, which had almost healed up. In these cases the agglutination titre was rather low, and it was only on the result of the deviation test that the animals were killed. The deviating properties of a serum seem therefore to be retained for a year and a half. The subcutaneous injection of mallein had no influence on the sera of glandered horses. The coefficient is rarely influenced, observations which correspond with those of *Miessner* and *Trapp*.

5. *The sera of healthy horses.*—The deviation coefficient in my experiments was often influenced by the previous injection of mallein, and therefore interfered with the accurate judging of the test. The mallein injection, as stated by *Miessner* and *Trapp*, has a pronounced influence on the sera of healthy animals, and this point is of considerable interest where the mallein test is made compulsory in outbreaks of glanders.

About 300 horses which were examined before and after malleining showed irregularities similar to those described by *Miessner* and *Trapp*, but the mallein used in these cases had not such pronounced action as mallein siccum (Foth) used by these gentlemen. About ten horses were specially examined to find if an increase of immune bodies occurs during a definite time after the injection of mallein, and how long they will be retained in the serum. Generally from the seventh to the tenth day after the injection the immune bodies could be traced in rather high doses (0·05); they were retained for about five to ten days, and disappeared rapidly afterwards, so that from the twentieth to the twenty-fifth day the original level was reached. Often such serum shows a

coefficient of 0·3 for over two months. The agglutination in these cases was in the proportion of 1 : 400, 1 : 600, 1 : 800, and 1 : 1000. It would be advisable not to use any mallein at all if the complement deviation test is used for diagnosis, but under prevailing conditions in South Africa this is impossible. Our instructions were therefore to collect the serum before the mallein injection was made, and should the two sero-logical reactions and the mallein reaction prove to be positive, the animal was to be destroyed for glanders. Up to the present I do not know of one case where a horse tested in this way, and subsequently killed, was found free from glanders. If any doubt occurred a second test, four weeks later, was made, at a time when all the influence of the previous mallein injection had disappeared, and when all tests were negative or when the serum tests were negative (viz., when no immune bodies could be detected in the serum) the animal was released from quarantine. By these means we have tested all the animals coming into the Institute, and up to the time of writing no outbreaks of glanders have occurred in the stables.

6. *The serum of sick equidae which are free from glanders.*—Several blood diseases have naturally an influence on the serum and its components, and it was therefore advisable to undertake experiments to prove how far the sero-diagnosis becomes affected by the different diseases of the country. The following tabulated statement shows these in a brief form :—

Disease.	Number of Cases.	Binding Coefficient.	Agglutination.	Remarks.
Strangles	6 Donkeys 2 Horses	—	1 : 200–500	Acute cases.
Horse-sickness ..	6 Horses	1 Horse 0·1, 5 others 0	1 : 300–600	Virus horses.
Piroplasmosis ..	2 Horses	0·05	1 : 400	*P. equi* present in the blood.
	2 Mules	0·1 & 0·05	1 : 200–500	„ „ „
	1 Donkey	0·2	1 : 200	Microscop., at the time no parasites present.
Piroplasmosis and horse-sickness	2 Horses	0·15	1 : 200–400	No parasites present.
Epizootic Lymphangitis	1 Horse	—	1 : 200	
Trypanosomiasis (*T. dimorphon*)	1 Donkey	0·1	1 : 300	Tryp. present.
	1 Mule	0·1	1 : 500	„ „

It can be seen that strangles, epizootic lymphangitis, and horse-sickness have no direct influence, but piroplasmosis has doubtless influenced the reaction, because the specific deviation test gave positive results when the agglutination test was negative. The serum in all cases was heated to 62° C., but this did not affect the non-specific bodies responsible for the deviation. All the sera had at the same time a slightly yellowish fluorescent appearance, and I am inclined to believe that the bile pigments are concerned in these deviations. Also in trypanosomiasis a similar effect could be observed. In practice a serum may be collected shortly after the time when the animal to be tested for glanders has passed through a piroplasmosis infection, and the test of the serum diagnosis may then give inaccurate results. It is therefore necessary that such sera should be submitted to the agglutination test before a final decision is given. In piroplasmosis of equidae we often found positive complement deviation which might have indicated glanders, but by means of the agglutination test or of a second complement test four weeks later all doubts

could be excluded. It would be very interesting to undertake investigations into the nature of those bodies which deviate the complement in a non-specific manner.

The sera of cats infected with glanders gave a positive complement deviation up to 0·1 c.c. serum (about four days after the infection). The exudates of the pleural cavities and of the pericardium, as we observed them in horse-sickness, contain no immune bodies, as was shown with several animals which died of horse-sickness while suffering at the same time from chronic glanders.

The Antigen.

1. *Extracts of glanders bacilli.*—The glanders cultures (twelve strains) used for my experiments were all isolated and kept at the original virulency by means of passage through cats. Two strains (Nos. 6 and 10) were especially useful as antigen and for the agglutination test. Following the notes of *Schütz* and *Schubert*, twenty-four hour old cultures (in Kolle's bottle) on glycerine-potato-agar were used. The cultures were killed at a temperature of 60° C., and washed off with saline water containing 0·5 per cent. carbolic acid (about 200 c.c. for one bottle). The contents were shaken for forty-eight to sixty-four hours at room temperature, and then centrifugalized for about a quarter of an hour. The clear upper part of the fluid was used for the tests in a dilution of one in one hundred. The results were constant with extracts prepared at different times. If kept on ice in dark bottles the extract was active for over two months. It is advisable not to use a fresh extract, as was stated by *Schütz* and *Schubert*.

2. *Extract from glanders organ.*—*Kayser* states in a paper that the glanders diagnosis from a dead animal by means of organ extract is possible. *Miessner* and *Trapp* state the contrary, because organ extract in a 4 per cent. solution with glanders serum never gave a deviation. I had similar results with organ extracts carried out on a large scale. In one case, however, the deviation was positive. An organ extract, derived from an extirpated submaxillary lymphatic gland from which a glanders strain was isolated, was mixed in small pieces with quartz sand, pounded in a mortar, diluted with physiological water about one in ten, and shaken for at least four days. This filtrate gave positive reactions with six different glanders sera when the extract was used in the dose of 0·5 c.c.

3. *Mallein.*—The preparation made here and the malleine brûte of the Institute Pasteur in a solution of 1 in 10 or 1 in 100, gave irregular results, and their use cannot therefore be recommended as antigen.

The Complement.

In the tests freshly collected guinea pig serum was always used. We easily obtained this serum by bleeding an animal to death. The blood was kept for fifteen minutes in the incubator, the coagulum was then detached from the wall of the test tube, and the tube kept on ice. By this means a considerable quantity of serum was always pressed out, and could be used for at least two days. If the complement, a most labile body, was kept under these conditions, no influence on its action could be observed. The complement must always be used in the smallest quantity which is sufficient to produce a complete haemolysis with the haemolysine. It is a well-known fact with every one who works with complement deviation that this is absolutely necessary, and therefore all the notes by *Schütz* and *Schubert*, and especially those of *Schuber*

are unnecessary. Every time we intended to make a complement test we tested the efficacy of the complement in the way shown in the following tabulated statement, and the smallest effective quantity was used.

Complement.			Haemolytic Amboceptor.	Sheep Blood Corpuscles 5 per cent.	Result.
		0·1 c.c.	1·0	1·0	Haemolysis.
0·75	Serum 1 : 10	0·0, 0·75 ,,	1·0	1·0	,,
0·6	,, ,,	0·06 ,,	1·0	1·0	,,
0·5	,, ,,	0·05 ,,	1·0	1·0	,,
0·45	,, ,,	0·045 ,,	1·0	1·0	,,
0·35	,, ,,	0·035 ,,	1·0	1·0	,,
0·25	,, ,,	0·025 ,,	1·0	1·0	Slight deviation.
0·15	,, ,,	0·015 ,,	1·0	1·0	Complete deviation.
0·1	,, ,,	0·01 ,,	1·0	1·0	,, ,,
		0·1 ,,	—	1·0	,, ,,
		—	1·0	1·0	,, ,,
		—	—	—	,, ,,

In each test-tube 3·0–4·0 c.c. physiological water.

The smallest quantity which gave complete haemolysis was therefore in this case 0·035 guinea pig serum, that is to say, for the test 96·5 c.c. physiological water was to be mixed with 3·5 guinea pig serum and 1 c.c. was used for each test tube. All naturally haemolytic systems, as pig serum with cattle blood, etc., and sera for complement tests were used.

The Haemolytic Amboceptor.

The rabbit sera used for our purposes were prepared by intraperitoneal injections of washed sheep and cattle blood corpuscles in ascendent doses. As soon as an animal showed a high titre (approximately 0·0025) it was chloroformed and the blood collected by bleeding the animal to death. The serum, collected under antiseptic precautions, was placed in small brown bottles, and these when sealed were kept on ice. They proved to be still useful after five months. The titre of these sera, viz., the haemolytic properties, did not diminish in any way. The hyperimmunization of the rabbits takes about eight weeks. I am inclined to think that the slow process of hyperimmunization increases the constancy of the amboceptor. That the resistance to light is very low, as was shown by *Miessner* and *Trapp*, was observed by me in several cases. The inactivation takes place at a temperature of 56° C. in a water bath, and the test for the titre was performed as usual, and is shown in the following table :—

Haemolytic Amboceptor.		Complement.	Sheep Blood Corpuscles.	Phys. Water.	Result.
1 : 100	0·25 = 0·0025	1·0	1·0	2·4	Haemolysis.
	0·15 = 0·0015	1·0	1·0	2·5	,,
	0·1 = 0·001	1·0	1·0	2·6	,,
1 : 1000	0·75 = 0·00075	1·0	1·0	2·0	,,
	0·50 = 0·0005	1·0	1·0	2·0	,,
	0·35 = 0·00035	1·0	1·0	2·0	,,
	0·25 = 0·00025	1·0	1·0	2·5	Slight deviation.
	0·15 = 0·00015	1·0	1·0	2·5	Complete deviation.
	0·10 = 0·00010	1·0	1·0	2·5	,, ,,
	—	1·0	1·0	3·0	,, ,,
	—	—	1·0	4·0	,, ,,

Kept for 1 hour at 37° C.

Result :—0·00025 is the haemolytic titre of the serum. For the experiments we used the double multiple.

The Red Blood Corpuscles.

This component of the test was not easy to keep constant, because it was rather difficult to obtain a constant amount of red blood corpuscles from an animal. The sheep suffer largely from anaemia due to oesophagostomum columbianum and other worms, and cattle also show variations in the number of red blood corpuscles. Blood corpuscles washed three or four times were generally used in a 5 per cent. emulsion, and compared with a standard tube containing a solution of haemolytic blood. This proved to be the best arrangement for the preliminary or orientation tests, and varying quantities from 5 to 10 per cent. were then added to make an emulsion of the blood corpuscles uniform for all tests. The emulsion was always prepared in physiological water of 0·85 to 0·9 per cent. (0·9 per cent. is isotonic for horse serum), and was kept on ice. Generally, such an emulsion should be kept and used for two days.

Conclusions.

The complement deviation method for the diagnosis of glanders proved to be very useful, and the results were better than those obtained with other methods. It was especially useful for the diagnosis of very recent and old cases of glanders. In conjunction with the agglutination test it enabled one easily to detect every horse suffering from glanders. The agglutination test was necessary as a supplementary method, because in sub-tropical and tropical countries blood diseases, and especially piroplasmosis, may have an influence on the reaction. The influence of mallein injection is similar to that caused by an infection, viz., causing the appearance of antibodies. The tests showed an increase of specific antibodies appearing from the sixth day after inoculation, which generally disappeared from three to four weeks afterwards. With the mallein used in my experiments (M'Fadyean) the increase of the specific antibodies was not very pronounced, and only of a short duration, so that it did not interfere with a second test four weeks later.

The method of *Schütz* and *Schubert* gave the best results, and embraces all the conditions for routine work. In a few cases, as was stated by *Kayser*, the watery extract of organs gave positive deviation with the serum of glandered horses. The coefficient (Bindungswert, *Miessner* and *Trapp*) was high for a long time (over one and a half years). To avoid reactions caused by non-specific bodies, which were especially demonstrated to be present in the sera of mules, the heating of the sera to the temperature of 62° C. was necessary. About 5 per cent. of the mules, however, still gave a non-specific deviation, and only the agglutination test could finally give a decision in these cases.

2. Agglutination Method.

This method has been used for a long period, and is well known. The publications of *Schütz* and *Miessner*, and lately from *K. Schütz* and *Sustmann*, contain all the necessary literature on the experiments carried out up to the present time. The method used by us followed strictly the indications given by *Schütz* and *Miessner*, and I therefore think that our results may be compared with all the statements made by these investigators.

Technique.

(a) *Suspension of glanders bacilli.*—Two glanders strains were especially used, because they proved to be easily agglutinable and their virulency was kept constantly at the same height by passing the strain through cats every four weeks. The culture was specially prepared on glycerine-potato-beef-agar,

and incubated for twenty-four to thirty-six hours at a temperature of 47° C. The cultures, after having been tested as to their purity, were heated to 60° C. during two hours. They were washed off with carbolized physiological water, and the turbid liquid filtered through ordinary filter paper. The filtrate was diluted until it had a slight milky appearance, and by looking through it in a standard tube small print could just be read. We kept a sealed standard tube in stock, and our filtrate was diluted by comparing with the standard tube. Every test liquid was proved before use with several sera having a well-known agglutination titre. Test liquids prepared in this manner could be used for a long time. Two c.c. were always added to a certain quantity of the serum which had to be tested.

(b) *Orientation test.*—As was proved in several experiments it was not necessary to test the sera in all dilutions. We examined therefore the agglutination properties in an *orientation test*, with six tubes for each serum. The serum was diluted 1 to 40 with carbolized physiological water, and then added to the tubes, as is shown in the next table :—

Test Tube.	Titre.	Quantity of Serum Dilution, 1 : 40.	Quantity of Testing Liquid.
1	1 : 100	0·8 c.c.	2 c.c.
2	1 : 200	0·4 ,,	2 ,,
3	1 : 300	0·27 ,,	2 ,,
4	1 : 500	0·16 ,,	2 ,,
5	1 : 800	0·1 ,,	2 ,,
6	1 : 1000	0·08 ,,	2 ,,

In cases where the complement test raised the suspicion of glanders, dilutions from 1 to 8000 were made, and a test was made with tubes with the following dilutions : 0 : 100, 1 : 200, 1 : 300, 1 : 400, 1 : 500, 1 : 600, 1 : 800, 1 : 1000, 1 : 1500, 1 : 2000, 1 : 4000, and 1 : 8000. The test tubes were kept for forty-eight hours at a temperature of 37° C., and the result was then noted. Agglutination was complete when the upper part became clear, and when on the bottom of the test tube an irregular star-like deposit had been formed. By shaking the tube fine irregular masses became suspended in the clear liquid. If no agglutination was present a round sharply localized deposit was found, and the contents of the test tube were turbid. By means of the centrifugal machine, following the instructions of *Miessner*, *Müller*, and *Pfeiler*, the agglutination could be accelerated, and the result of the test obtained in half an hour (1000 revolutions in a small electric centrifugal machine). We used this method very rarely. Generally speaking, horses with an agglutination from 1 : 100 to 300 were considered free from glanders ; an agglutination titre from 1 : 1000 and more was found in horses with glanders. Animals with a titre 1 : 500 up to 1 : 1000 were suspected of glanders. It is impossible to give a definite mark for the differentiation between specific and non-specific agglutination, because in rare cases agglutination in 1 : 1000 to 2000 was observed and the animals proved to be free from glanders ; therefore, horses which give a constant agglutination titre from 1 in 200 to 1 in 1000 in several tests were considered free from glanders.

In our case the definite decision was made dependent on the complement test or on a second similar test three weeks later. If then the agglutination

titre was the same, and the complement test negative, the animal was discharged and declared to be free from glanders. As this work was only used as a complementary test in our routine no further experiments were carried out, because in all the doubtful cases, where the complement test did not allow of a definite decision, the agglutination test gave a different result, and when the contrary was observed the complement test superseded the agglutination test. Especially in all cases of piroplasmosis, or in those where non-specific reactions were common, the agglutination test allowed of a definite diagnosis in these. The case of two mules proved interesting. The latent power of infection was rather long and reached almost ten days. It was observed in this experiment that the agglutinine appeared later than the complementophile substances, which could already be traced on the sixth day after infection. It was proved that mallein also had a certain influence on the height of the agglutinating titre, but the specific bodies seemed to disappear rather quick by a parallel observation already made in the complement test. The different diseases common in the country had no influence on the agglutination titres, which proved to be within the expected limits, and especially in several experiments on piroplasmosis no influence could be observed. The agglutination titre of the glandered horses was as follows :—

Of twenty-seven equidae :

0	had an	agglutination	titre of from 1 : 100 to 300.
2	do.	do.	1 : 100.
1	do.	do.	1 : 500.
5	do.	do.	1 : 600.
6	do.	do.	1 : 800.
8	do.	do.	1 : 1000.
2	do.	do.	1 : 1500.
2	do.	do.	1 : 2000.
1	do.	do.	0 : 4000.

In my observation the influence of external causes on the agglutination corresponded with the well-known fact described in all the publications on this subject.

(c) *Precipitine test.*—By means of this method precipitines, as special bodies, can be demonstrated in the immune sera of animals suffering from glanders. It was *Wladimiroff* who first described precipitine actions in the serum of glandered horses. By mixing sera with a filtrate of glanders bacilli specific deposits could be produced but which in the serum of healthy animals were never present. Similar results were described by *Bonome* and *Müller*, and lately *Pfeiler* tried to use the precipitine method for the practical diagnosis of glanders. Also *Miessner* was engaged on this question, and he observed typical precipitine rings when bacilli of glanders extract or malleinum siccum Foth (1 : 10) was poured on to the serum of glandered horses. For our experiments we had no malleinum siccum at our disposal, and all the other mallein preparations were useless ; they contained a certain amount of glycerine. All the tests were carried out with a filtrate of glanders bacilli in horse serum with 0·5 per cent. of carbolic acid. Before use this liquid was diluted six to twelve times with normal horse serum. The tests were made in small test tubes containing 0·3 c.c. of the serum to be tested, and the same quantity of extract was added. A positive reaction was present when a colourless, round, disc-like zone was formed at the surface where serum and extract came in contact. Sufficient controls with normal sera always had to be made. Different glanders sera, five to twenty minutes after their addition to extract, always gave a well pronounced ring, but similar precipitation symptoms were also observed

in about ten of our hundred normal sera. The test was repeated several times with different extracts, and all sources of error were taken into consideration, but always with a similar result, and I am inclined to believe that non-specific bodies are responsible for these reactions. From a practical point of view the method may be of use for the purpose of detecting glandered horses, but the technical difficulties which favour many errors, together with the fact that here about 10 per cent. of horse sera gave non-specific precipitine rings, prevent the method from ever coming into use as a simple independent test. It would be of great interest for further investigations in this direction in order to identify the nature of the bodies which are engaged in this non-specific reaction. The co-operation of this third method with the other two does not seem to have a practical importance, because the agglutination and the complement deviation tests assist in the diagnosis of both recent and old cases of glanders.

Conclusions.

The complement and the agglutination methods, with the technique described by *Schütz* and *Schubert*, *Miessner* and *Trapp* on the one hand, and *Schütz* and *Miessner*, *Schütz*, *Sustmann*, and others on the other hand, gave results which allow of the diagnosis of occult glanders more easily than the mallein test. The precipitine method gives positive results in all cases of glanders, but in the serum of healthy animals, in about 10 per cent. of all cases investigated, reactions were also observed. As these were non-specific reactions this method cannot be used independently until other experiments have shown a way for excluding them.

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