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Heartwater in Sheep.—The Weil-Felix reaction and an investigation into the bacterial content of the blood with particular reference to the use of "K" medium.

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THE WEIL-FELIX REACTION.

THE existence of a common antigenic factor [Castanida and Zia (1933), White (1933)] in *Rickettsia* and *Bacillus proteus* X is known to be responsible for the specific O agglutination of *B. proteus* X by typhus serum, originally described by Weil and Felix and now known universally as the Weil-Felix reaction.

Attention was again focussed on this reaction when Felix and Rhodes (1931) confirmed the observation of Fletcher and Lesslar (1925) that there existed two serologically distinct types of tropical typhus which reacted specifically with *B. proteus* X19 on the one hand and *B. proteus* XK on the other. An additional interesting feature of this finding was that *B. proteus* XK was merely a non-indologenic variant of X19, and had been obtained inadvertently during the course of routine subcultivation.

Further investigation into the serological types of typhus virus has led Felix (1933) to correlate " the agglutinogenic and immunogenic properties of different types of virus ". This has led to the suggestion that there is a variant of *B. proteus* X that corresponds serologically with each of the *Rickettsiae* causing the many different diseases of the typhus group.

Cowdry (1926) showed that a Rickettsia, *R. ruminantium*, is the causative agent of heartwater, and consequently the possibility of demonstrating a positive Weil-Felix reaction with one or other of the *proteus* OX strains was considered, since the establishment of such a reaction would be of the greatest value in the further study of the disease.

Our colleague, Dr. E. M. Robinson, informed us (personal communication) that previously he had investigated this reaction in heartwater-affected and -recovered sheep but with negative results. Nevertheless we decided to take up the problem anew using—

(*a*) A larger number of sera from sheep in various stages of the disease, that is during the reaction, at various periods after recovery and after "hyperimmunization" by several injections of virulent blood.

(*b*) Antigens consisting of single cell cultures of OX2, OX19, and OXK in the hope of the chance isolation of a specific variant as was the case with OXK.

(*c*) Antigens consisting of cultures of organisms isolated from sheep infected with heartwater.

(*a*) The initial *proteus* strains used were cultures of OX2, OX19, and OXK, received through the courtesy of Dr. A. Felix, to whom we wish to express our thanks. To serve as controls to these cultures we prepared agglutinating sera in rabbits by repeated intravenous injections of saline suspensions killed by heating at 60° C. for half an hour, and in goats by intravenous injections of saline suspensions of single cell cultures killed with 0.1 per cent. formalin. Cross agglutination tests were carried out with living suspensions in saline of approximately the same density; the volume of fluid in each tube was made up to 2.0 c.c. with saline and readings were taken after two hours at 45° C. and overnight storage at room temperature (22°-26° C.). The results are recorded in Table I.

TABLE I.

Antigens.	Agglutinating Serum.					
	OX2 (s.c.)	OX19 (s.c.)	OXK (s.c.)	OX2 (orig.)	OX19 (orig.)	OXK (orig.)
OX2 (s.c.)...	1,280 (3)	40 (2)	80 (3)	1,280 (3)	80 (1)	20 (1)
OX19 (s.c.)	20 (1)	10,240 (3)	40 (2)	20 (0)	2,560 (3)	20 (0)
OXK (s.c.)..	40 (1)	40 (1)	10,240 (2)	20 (0)	20 (0)	5,120 (2)
OX2 (orig.).	2,560 (1)	40 (2)	80 (1)	1,280 (3)	20 (1)	20 (1)
OX19 (orig.)	40 (0)	10,240 (1)	80 (1)	20 (1)	5,120 (2)	20 (0)
OXK (orig.)	40 (1)	40 (2)	10,240 (2)	20 (0)	20 (0)	10,240 (2)

s.c. = single cell culture. orig. = original culture.

(3), (2), etc. = degrees of agglutination, (3) being complete. Double the dilution noted was negative or trace, and half was complete (3). The lowest dilution used was 1/20.

(*b*) *Agglutination of Single Cell strains of* Proteus *OX.*— Mention has been made of the chance isolation of a serologically distinct mutant of OX19, which reacts specifically with the sera of scrub typhus patients. By the use of this variant OXK and OX 19 scrub typhus may be differentiated from shop typhus, two types of the tropical disease encountered in Malaya and the Dutch East Indies. Therefore it was considered possible that by isolating

from the original *proteus* cultures a large number of single bacilli a variant might be obtained which would be agglutinated by heartwater sera, the assumption being made that there was present an agglutinable variant, but in such small numbers that its agglutination was marked by the much greater number of non-agglutinable organisms. It was realized that if such a variant did exist its isolation would be a matter of pure chance unless many thousands of single-cell cultures were examined. A compromise was made by obtaining 50 single cell cultures from OX2, OX19, and OXK. The resulting 150 antigens were tested against normal sheep sera and heartwater immune sera.

None was agglutinated by any serum at a dilution of 1-40.

(*c*) *Attempt to Isolate a* Proteus *X or other Serologically Similar Organisms from Heartwater Sheep.*—It is the routine practice at these laboratories to maintain the virus of heartwater by passage through sheep. Ample material was available therefore for an attempt to isolate a specific *proteus* or other organism which might have some relation to the heartwater virus. Other authors [Anigstein (1933), Kuczynski (1927), and Martin (1931)], working with diseases of the typhus group, have been able to isolate from various patients strains of *proteus* X which have been shown to have a definite relation with the disease in question.

Kendall (1931) reported that, by the use of his medium, non-filterable bacteria had been rendered filterable, and, further, the inoculation of this medium with apparently bacteriologically sterile blood from influenza patients had resulted in the isolation of a coccus which possessed a definite relation to the virus. We were further stimulated in our investigation by the report of Hadley, *et alia* (1931), in which Shiga's dysentery bacillus was shown to have a filterable stage.

Our main object in undertaking the work was in the hope of isolating a germ which would be agglutinable by heartwater serum, or which could be used as an antigen in the prevention of the disease. As our results were entirely negative, we consider it necessary to record only the main points, omitting details.

Methods.

In all, material from 51 sheep was investigated—of these 25 were infected with heartwater, 13 with blue tongue, 5 with enteritis [cause unknown (?) dietetic], and 8 were normal animals. All were Merinos, the majority castrated males and the ages varied from 1 to 5 years. The blood of 20 heartwater sheep (87 bleedings), 8 normal sheep (17 bleedings), 13 blue tongue sheep (25 bleedings), and of 5 "enteritis" sheep (5 bleedings) was cultured. In 11 heartwater sheep, scrapings from the jugular vein were cultured, and in four instances the urine. In some cases the blood, jugular scrapings, and the urine from the same sheep were investigated.

Collection of Blood.

Prior to commencing the investigation, an attempt was made to work out a method of obtaining blood in a sterile manner. The method used is given by Mason (1934), and, in brief, consisted in

closely clipping the wool from the jugular region, the application of absolute alcohol and then ether to this area, the insertion, with one thrust, of the needle into the vein, the allowing of 30.0 c.c. of blood to escape, and finally the collection of the sample in a sterile tube.

Jugular Scrapings and Urine.

The sheep was pithed and with sterile precautions, the jugular vein (cervical portion) exposed, and about 20.0 cm. dissected out. The intima was exposed and thoroughly scraped with a knife, the scrapings being transferred to one or other medium. Urine was obtained direct from the bladder by means of a bulb pipette, aseptic precautions being adopted.

Media.

1. Ordinary beef infusion peptone broth.

2. Clot broth. About 10.0-12.0 c.c. of blood was allowed to clot in a test tube and the serum discarded. Broth was then added to replace this serum.

3. Horse flesh infusion peptone agar, plus 5 per cent. of a mixture of equal parts of sheep serum and sheep haemolysed red cells [Mason (13)]. This medium was used in the large flat-bottomed tubes described by Mason (14), and was always incubated for three days at 37° C. prior to use.

4. Litmus lactose agar in the tubes noted under (3).

5. Kendall (K) medium. Three separate lots prepared from sheep gut according to Kendall, and one sample obtained from Difco were used.

6. "E.B." medium prepared from sheep brain according to Eberson and Mossman (15).

7. Robertson's meat broth (horse flesh) medium.

All media were autoclaved for half-an-hour at 120° C. prior to use. [In the case of medium (3) the agar was autoclaved and having cooled to 45°-50° C. the serum-haemolysed-cells mixture was added.]

General Scheme.

When blood was investigated, bleedings were usually made at the first rise of temperature and then at 1 or 2 days intervals until the death of the animal. When broth was used from 0.5 c.c. to 1.0 c.c. of blood was pipetted into 10.0 c.c.-15.0 c.c. of medium, and this incubated at 37° C. for 14 days; a sub-culture was then made into fresh broth, this incubated for a further 7-14 days, and finally a further sub-culture made on serum-haemolysed-cells-agar or Hauduroy's (19) serial plating method on either serum-haemolysed-cells-agar or litmus lactose agar was carried out.

Blood clots were incubated at 37° C. for one month (reduction in volume due to evaporation being made up with sterile distilled water), when a smear was made on serum-haemolysed-cells-agar, and this incubated for 48 hours.

K medium (30.0 c.c.-50.0 c.c. with 0.5 c.c.-1.0 c.c. of blood) was incubated at 30° C. or 37° C. for 14-28 days. Two further sub-cultures (0.5 c.c.-1.0 c.c. into 10.0 c.c.-15.0 c.c. medium) were made at 10-14 days interval. If no growth was demonstrable (either by naked eye or by stained smear) Hauduroy's serial plate procedure on serum-haemolysed-cells medium or litmus lactose agar was adopted, from 3-8 such platings being conducted.

Throughout, smears stained by Giemsa's and Gram's methods were examined even in apparently negative tubes.

Special attention was given to any tube, which, by stained smear examination, showed what could be developmental forms of bacteria. Serial platings of such material were carried out as many as 25 times before a negative result was accepted.

Every micro-organism which grew, with the exception of obvious contaminants from the sheep's skin or from the air was investigated. This consisted in (1) carrying out with every germ agglutination tests with the heartwater sera previously mentioned, (2) in some cases injecting a K culture or a saline suspension of the germ intracerebrally and intraperitoneally and sometimes intra-testicularly into guinea pigs and mice and subinoculating the brains of such animals intracerebrally into fresh guinea pigs and mice, and (3) injecting living K culture and saline suspensions of the microbes subcutaneously and intravenously into sheep and later testing with virulent blood for immunity against heartwater.

Organisms which developed quickly, within 1-3 days, in the original culture tube, and which on further examination proved to be aerobic Gram positive spore-forming bacilli, staphylococci, large copiously growing aerobic Gram positive bacilli (usually pigment producers) were considered to be contaminants and were discarded. All diphtheroids (of which a considerable number was isolated) and Gram negative bacilli were retained and examined in some detail; in addition to the tests already mentioned, the Gram negative bacilli were investigated biochemically.

Results.—These may be briefly summarised under five headings:—

(1) No *proteus* X-like bacillus was isolated.

(2) No germ, which was isolated, was agglutinated by heart-water or *proteus* OX sera at a titre significantly higher than that obtained with normal serum.

(3) It was not possible to produce a transmissable disease, symptom or reaction in guinea pigs or mice by the intracerebral, intraperitoneal or intratesticular injection of living K culture or saline suspension of 8 organisms obtained from 6 sheep.

(4) K cultures and saline suspensions of 14 germs from 12 sheep injected intravenously and subcutaneously (simultaneously) into 28 sheep, apart from a transitory rise in temperature, produced no reaction. Sheep, treated with two such inoculations at from 2-3 weeks interval, were not immune to heartwater, when tested 10-20 days later with intravenous injections of virulent blood.

(5) Formol-killed saline suspensions of *proteus* OX2, OX19 and OX2 (two subcutaneous injections at 14 days interval) did not immunise sheep against heartwater (as tested by the intravenous injection of virulent blood).

Discussion.

In Appendix 1 a résumé is given of the treatment adopted with each sheep and of the germs isolated therefrom. It will be noted that from the heartwater sheep the following germs were isolated: 22 diphtheroids (small Gram positive rather sparsely growing bacilli), 11 staphylococci (white or yellow), 7 Gram negative bacilli (*B. cloacæ*, *B. pyocyaneus*, a member of the alkaligenes group, and three unidentified bacilli, not members of the colontyphoid or *proteus* groups), two tetrads, two streptococci, and 10 Gram positive copiously growing aerobic bacilli (8 sporers), and one Gram negative coccus. From the 26 remaining sheep (normal, blue tongue and "enteritis") four diphtheroids, one staphylococcus and one Gram positive aerobic sporer were obtained. Although the number of bleedings is not large enough to lend itself to accurate statistical analysis, it is interesting to note that basing results on the number of bleedings, 6 of 37 (16.2 per cent.) of the non-heartwater bleedings gave positive growth results, and 55 of 87 (63.2 per cent.) of the heartwater bleedings and jugular scrapings were positive. Reckoning only diphtheroids, the figures become 4 of 37 (11 per cent.) and 22 of 87 (25 per cent.).

We are of the opinion that possibly in non-heartwater sheep the figure 11 per cent. for diphtheroids is actually too high. This is based on results obtained in similar but not identical investigations (unpublished) on the sterility of the blood of normal sheep. These results showed that blood taken direct from the heart of a killed lamb or sheep was sterile; the occasional growth obtained was obviously of aerobic origin.

We are unable to state if the diphtheroids (or other germs) obtained from the blood of heartwater sheep have actually any connection with the virus of the disease. There is no result which would indicate any relation, but the possibility does exist that one or more might have been a developmental stage of the virus, but not at any time obtained by us in a phase capable of being specifically agglutinated or of setting up disease symptoms in laboratory animals or sheep. However, we consider it more likely that they had their origin in the intestine and were able to escape easily into the blood stream through the inflamed gut. This opinion is strengthened by the fact that the highest percentage of positive blood cultures was obtained when the disease was well advanced, bleedings taken prior to the rise in temperature usually being sterile.

One germ only deserves a somewhat detailed description; its growth characteristics, the apparent developmental phase through which it went, and its pleomorphism gave us cause for hope that we had obtained a bacillary stage of the causative rickettsia.

Sheep 34443.—Injected 17/10/32 with 10.0 c.c. of virulent blood from sheep 34910. This latter animal died from and showed the typical lesions of heartwater on 28/10/32. On 24th October,

1932, 1.0 c.c. of blood was sown in 30.0 c.c. of K medium, and incubated at 37° C. After four days the medium was slightly cloudy, but a Giemsa smear showed nothing to arouse suspicion. Sub-cultures on serum-haemolysed-cells-agar, incubated aerobically and anaerobically remained apparently sterile. A Giemsa smear from the original K tube made on 3rd November, 1933, revealed a large number of tiny red-blue bacilli, which when they occurred in clumps, closely resembled *rickettsiae*. Aerobic and anaerobic sub-cultures on agar remained without visible growth. Hauduroy's technique on litmus lactose agar was now adopted and on the third passage (24 hours incubation between each) what appeared to be Hadley's G colonies were obtained. These were so tiny as to be visible only by the aid of a lens when the tube was held in a certain position in relation to the source of light. Giemsa smears showed a very pleomorphic organism; every gradation between a rickettsia-like microbe and a medium-sized bacillus was seen, but the predominant form was what might be termed " knobbly ", i.e. cocci showing protuberances, tapering bacilli ending in swellings or nodular-curved rods.

At this stage the germ would not grow on " dry " serum agar or in ordinary broth, but did so on the surface of " flooded " agar. The serial passages were continued 13 times, at which stage the germ grew reasonably well—sub-cultures on " dry " agar or in broth gave, after 2-4 days' incubation at 37° C., a scanty, rather dry grey growth or a faint turbidity. Smears made at this time revealed a bent or curly Gram positive bacillus, still " knobbly ", and only a very occasional coccus, or rickettsia-like organism. Cultures in K medium, broth, serum-haemolysed-cells-agar and litmus lactose agar made during a period of six months did not result in any further change in morphology or type of growth. The bacillus was not agglutinated by heartwater or *proteus* OX sera at a dilution of 1/40. K cultures (2 and 10 days at 30° C. and 37° C.) and saline suspensions from " flooded " agar injected intraperitoneally, intracranially and intesticularly into guinea pigs caused no disease; the brains of the intracranially injected animals passaged into fresh guinea-pigs (and these later again passaged) gave negative results. The same technique in mice (omitting the intratesticular route) gave the same negative results. As appendix shows, sheep were not demonstrably affected by the intravenous and/or subcutaneous injection of K culture or saline suspensions and were not later shown to be immune to heartwater.

Conclusions.

(1) The H and O variants of *proteus* X2, X19 and XK are not agglutinated by serum from sheep, affected with, recovered from or " hyperimmune " to heartwater.

(2) None of 50 single-cell cultures (150 in all) from the O variants was agglutinated by these sera.

(3) Blood cultures from sheep infected with heartwater gave more positive growth results than did blood from normal sheep or from sheep affected with blue tongue or an enteritis of unknown

origin. A considerable percentage of the organisms isolated probably had their origin in the intestine and escaped via the inflamed gut into the blood stream.

(4) Of the microbes investigated, none was specifically agglutinated by heartwater or *proteus* OX sera. In addition none set up disease symptoms in guinea pigs, mice or sheep, and sheep injected twice with living cultures were not protected against heartwater.

REFERENCES.

ANIGSTEIN, L., 1933. Observations concernant la variabilité des souches cultivés du typhus tropical. *C.R. Soc. Biol.*, Vol. 112, p. 1449.

CASTANIDA, M. R., AND ZIA, S. (1933). The Antigenic Relationship between *Proteus* ×19 and *Typhus Rickettsiae*. A study of the Weil-Felix Reaction. *J. of Exp. Med.*, Vol. 158, No. 1, pp. 55-62.

COWDRY, E. V. (1926). Cytological Studies on Heartwater, 1. The Observation of *R. ruminantium* in the Tissues of Affected Animals. *11th and 12th Reports of the Director of Vet. Ed. and Res., Union Dept. of Agric.*, pp. 161-180.

COWDRY, E. V. (1926). Cytological Studies on H.W. 2. *R. ruminatium* in the Tissues of Affected Animals. *11th and 12th Reports of Dir. of Vet. Ed. and Res., Union Dept. of Agric.*, pp. 181-198.

EBERSON, F., AND MOSSMAN, W. G. (1932). Procedures in the Isolation and Cultivation of an Organism from Berkefeld N. Filtrates of Poliomyelitis virus. *Procs. Soc. Exper. Biol. and Med.*, Vol. 30, No. 1, pp. 92-97.

FELIX, A., AND RHODES, M. (1931). Serological Varieties of Typhus Fever. *J. of Hyg.*, Vol. 31, No. 2, pp. 225-246.

FELIX, A. (1933). Serological Types of Typhus Virus and Corresponding Types of Proteus. *Trans. of Royal Soc. of Trop. Med. and Hyg.*, Vol. 27, No. 2, pp. 147-172.

FLETCHER, W., AND LESSLAR, J. E. (1925). *Bull. Inst. Med. Res. Fed. Malay States*, No. 2.

HADLEY, P.; DELVES, E.; AND KLUNEK, J. (1931). The Filterable Forms of Bacteria: A Filterable Stage in the Life History of the Shiga Dysentery Bacillus. *J. Inf. Dis.*, Vol. 48, pp. 1-159.

HAUDUROY, P. (1929). Les ultravirus et les formes filtrantes des microbes. Masson et Cie, Paris.

KENDALL, A. I. (1931). Observations upon the Filterability of Bacteria, including a Filterable Organism obtained from Cases of Influenza. *Science N.S.*, Vol. 74, No. 1910, pp. 129-139.

KUCZYNSKI, M. H. (1927). Die Erreger des Fleck und Felsenfiebers. J. Springer, Berlin.

MARTIN, P. H. (1931). *Annual Report, Inst. Med. Res. Federated Malay States*, p. 48.

MASON, J. H. (1934). A Note on the Cultivation of Anaerobes. *This Journal.*

MASON, J. H. (1933). A New Culture Tube. *J. S.A. Vet. Med. Assoc.*, Vol. 4, No. 2, pp. 89-90.

WHITE, P. BRUCE (1933). The O-Receptor Complex of *B. proteus* × 19. *Brit. J. of Exp. Path.*, Vol. 14, No. 3, pp. 145-148.

APPENDIX 1.

("Not agglutinated" means that the organisms were not agglutinated by heartwater or *proteus* OX serum at a dilution of 1-50.)

Sheep 31281.—Bled five times, at daily intervals, during fever. Blood allowed to clot, serum decanted, and clots incubated aerobically and anaerobically. Sub-cultures on serum agar and serum broth put up daily from depth of clot and these incubated aerobically and anaerobically. The first two bleedings sterile; from remainder a streptococcus, large Gram negative bacillus, small Gram negative bacillus, and a diphtheroid isolated. The bacilli not agglutinated.

Sheep 31763.—Bled three times during fever, as clot broths. A staphylococcus, a Gram positive aerobic sporer and a pleomorphic diphtheroid isolated. Last germ not agglutinated.

Sheep 31098.—Bled six times during fever, as clot broths and into meat broth. First two and fifth bleedings sterile. The third, fourth, and sixth bleedings positive in 1-3 days. *B. pyocyaneus*, *B. cloacae*, member of alkaligenes group, Gram negative bacillus (not colon-typhoid or *proteus* group) staphylococcus and a dipththeroid. None agglutinated.

Sheep 32120.—Bled seven times during fever as clot broths. The third, fifth, and seventh bleedings sterile. Bleeding 1 positive 9 days Gram positive sporer; bleeding 4 staphylococcus 14 days; bleeding 6 Gram positive sporer 17 days; bleeding 2 by Hauduroy's technique diphtheroid—not agglutinated and non-pathogenic.

Sheep 32118.—Bled nine times prior to and during fever as clot broths. First, second, fourth, and ninth bleedings sterile. From remainder a staphylococcus and Gram positive sporer. Pithed at height of fever. Jugular scrapings into its own clot broth, clot Tyrode and into clot Tyrode and clot broth from a normal sheep. One (its own) clot broth positive 10 days, Gram positive sporer. Remainder sterile.

Sheep 31720.—Pithed at height of fever. Jugular scrapings into normal sheep clot broth and its own clot broth, and in addition a clot broth put up. All tubes sterile after 14 days. With Hauduroy's technique a fine diphtheroid isolated from one tube. After one month's sub-culturing this grew as a profuse, yellow paint-lifle growth. Non-agglutinated.

Sheep 32049.—Bled eight times prior to and during fever. The first three bleedings sterile. From remainder after 3-8 days, a Gram positive sporer, a streptococcus, a staphylococcus and a fine diphtheroid obtained. The diphtheroid not agglutinated.

Sheep 33518.—Bled out at height of reaction. Clot broths made, clot broths inoculated with jugular scrapings and clot broths from normal sheep clot broth inoculated with scrapings. A Gram negative bacillus (not colon-typhoid or *proteus* group), a Gram negative coccus and a Gram positive cocco-bacillus isolated. The first germ not agglutinated.

Sheep 34103.—Pithed at height of reaction. Clot broths made; jugular scrapings into normal sheep clot broth, urine into meat broth and normal sheep clot broth. The normal sheep clot broth plus scrapings positive eight days with diphtheroid. Not agglutinated. Remainder sterile.

Sheep 34607.—Pithed at height of reaction. Jugular scrapings into its own clot broth, urine into its own clot broth, meat broth and broth. Urine-broth, positive three days with staphylococcus, scrapings—clot broth positive four days Gram positive sporer.

Sheep 34103.—Pithed at height of reaction. Jugular scrapings into normal sheep clot broth and urine into broth, meat broth and normal sheep clot broth. A clot broth made from heart blood. Scrapings and urine sterile. Clot broth gave a fine Gram negative bacillus and a fine diphtheroid. Neither agglutinated.

Sheep 34095.—Pithed at height of reaction. Jugular scrapings into meat broth—positive seven days Gram positive sporer, blood into meat broth—positive four days staphylococcus, urine into broth—positive four days staphylococcus.

Sheep 34910.—Bled twice during fever into K medium and into broth. Ks at 30° C., broths at 37° C. First K bleeding sterile (serially passaged on litmus lactose agar nine times; second K bleeding apparently sterile on sixth serial passage. The fifth and sixth passage tubes sealed off and set aside for six weeks at room temperature. A diphtheroid (good growing) growing on fifth tube and a tetrad on the sixth. Neither agglutinated.

The broths treated as the Ks. Both sterile.

Sheep 34054.—Bled six times during fever as 34910.

Ks: First, fourth, fifth, and sixth bleedings sterile. From the second and third a tiny diphtheroid isolated. Not agglutinated.

Broths: The first five bleedings incubated at 37° C. for from 25 to 28 days. Subcultures in K incubated at 37° C. for one month and smears made on agar—all sterile. The sixth bleeding positive three days—staphylococcus.

Sheep 3443.—Bled six times during fever as 34910.

Ks: By mistake, all but one bleeding 24.10.32 (the third) discarded. For description of germ obtained see text.

Broths: From the first three bleedings a staphylococcus, and from the fifth a Gram positive sporer obtained; the fourth and sixth sterile after one month. The positive results were got in the original tubes after 7-14 days incubation.

Sheep 35024.—Bled five times during fever as 34910.

Ks: The second, third, fourth, and sixth bleedings sub-cultured at 14 days interval, four times through K and then serially plated three times; all negative. First bleeding, good growing diphtheroid isolated from original tube after 17 days. Not agglutinated. Fifth bleeding—poorly growing diphtheroid from original tube after 12 days. Not agglutinated.

Broths: Original tubes negative after one month; sub-cultnes on agar negative after one week.

Sheep 35029.—(Injected i.v. with 35.0 c.c. of a 24 hours K culture of diphtheroid isolated from the third K tube of 34051. No reaction. After nine days bled into K and broth.)

K: By serial platings a Gram positive, fine granular bacillus isolated. On smear, the organism often appeared to be made up on tiny granules. Not agglutinated.

Broth: Sterile after one month.

Sheep 35007.—Bled thirteen times prior to and during fever as 34910.

Ks: The first to seventh, ninth, eleventh, twelfth, and thirteenth bleedings sub-cultured through K three times at 14-day-intervals. Then serially plated three times—all negative. From the eighth and tenth fine diphtheroids isolated—neither agglutinated.

Broths: All incubated one month and then sub-cultured on agar for three days. All negative, except the second—a large, good growing Gram positive bacillus.

Sheep 35533: Bled seven times during fever as 34910.

Ks: The first, third, fifth, sixth, and seventh bleeding incubated for one month, then serially plated three times—all negative. From the fifth a copiously growing Gram positive bacillus isolated from original tube after four days incubation. From the fourth a fine diphtheroid obtained. Not agglutinated.

Broths: All incubated 25 days—then into fresh broth for 10 days—then on agar—all negative.

Sheep 34894.—(Received 90·0 c.c., intravenously, of a 24 hours K culture of Gram positive bacillus isolated from 34443, 24/10/32. No reaction.)

Nineteen days later bled into K. Sub-cultured into fresh K in 7 days, into EB in a further 16 days, and 2 days later serial plating commenced. By this technique a rather stout vacuollated "knobbly" bacillus isolated. Grew very poorly. Not agglutinated.

Sheep 34761.—Bled three times during fever as 34910.

Ks: Incubated 20 days, then into fresh K for 7 days—serial platings three times. The second, third, and fourth bleedings sterile. From the first a very profusely growing Gram positive bacillus.

Broths: Incubated one month—then into fresh broth for 10 days—all negative.

Sheep 34177: Pithed at height of reaction and jugular scrapings cultured in K and broth.

K: Positive in 8 days—staphylococcus.

Broth: Incubated for 14 days—then into fresh broth—negative 14 days.

Sheep 35829.—Pithed at height of reaction and jugular scrapings cultured into K and broth.

K: Incubated 10 days: then into fresh K for 10 days: sown on agar—staphylococcus.

Broth: Incubated 10 days: then in fresh broth. Negative 14 days.

Sheep 35035.—Treated as 35829.

K: A "knobbly" diphtheroid isolated. Not agglutinated.

Broth: Negative.

Sheep 34871.—Treated as 35829.

K: A profusely growing Gram positive bacillus isolated. Not agglutinated.

Broth: A staphylococcus isolated.

Normal Sheep.

Seven normal sheep (35803, 35808, 34806, 34998, 34093, 35819 and ×) bled into K and broth as 34910.

Ks: Incubated for 14 days; then sub-cultured into fresh K and incubated for a further 14 days. Then cultured in "flooded" agar for seven days (evaporated fluid replaced as required with sterile distilled water). All negative.

Broths: Incubated for 14 days; then into fresh broth for 14 days. All negative.

Sheep Y: Ten clot broths made at one bleeding. Incubated for one month. Two tubes positive after four days incubation, with apparently the same fine growing diphtheroid.

Blue Tongue Sheep.

Sheep 34681, 34713, 34751.—Bled at height of temperature reaction into meat broth and as clot broths. The first positive in four days—staphylococcus. Remainder sterile after 20 days' incubation.

Sheep 34562, 34633, 34750, 34581, 34522, *and* 34626.—Bled as clot broths at height of temperature reaction. The first positive in 10 days—fine diphtheroid. Remainder negative after 20 days.

Sheep 35007, 35003, 35033, 35006.—Bled during fever on each of three successive days into meat broth and broth. All incubated 20 days. The first positive (in meat broth only) with a fine growing diphtheroid.

Sheep 35001, 35035, 35011.—Bled during fever into broth and meat broth. All negative after one month's incubation.

"Enteritis" Sheep.

Sheep 34356, 34547, 34717, 34653, *and* 33961.—Bled as clot broths and into broth. The first positive with a Gram positive sporer after four days. Remainder negative in 20 days.

APPENDIX 2.

Summary of the inoculations of living cultures and suspensions into sheep with the result of the immunity test with virulent heartwater blood:—

Sheep.		*Treatment.*	*Immunity Test.*
32957	22/3/33	10.0 c.c. s.c. saline suspension of fourth bleeding diph. of 35533	18/4/33 died H.W.
	5/4/33	25.0 c.c., ditto.	
32952	22/3/33	10.0 c.c. s.c. saline suspension of diph. of 34443	Do.
	5/4/33	20.0 c.c., ditto.	
32958	22/3/33	10.0 c.c. s.c. saline susp. of Gram pos. bacillus of 34871	Do.
	5/4/33	20.0 c.c., ditto.	
32949	22/3/33	10.0 c.c. s.c. saline susp. of diph. of 35035	Do.
	5/4/33	20.0 c.c., ditto.	
33117	22/3/33	10.0 c.c. s.c. saline susp. of staphylococcus from 34177	Do.
	5/4/33	20.0 c.c., ditto.	
33118	22/3/33	10.0 c.c. s.c. saline susp. of staphylococcus of 35829	Do.
	5/4/33	20.0 c.c., ditto.	
32948	22/3/33	10.0 c.c. s.c. saline susp. of grain pos. bacillus of 35029	Do.
	5/4/33	20.0 c.c., ditto.	
32507, 33119, 33104	22/3/33	10.0 c.c. s.c. of formol-killed saline susp. of OX2, OX19, and OXK respectively	All died H.W.
	5/4/33	20.0 c.c., ditto.	
35803	9/1/33	2.0 c.c. i.v. 15.0 c.c. s.c. saline susp. of bacillus of 34894	15/2/33 died H.W.
	27/8/33	5.0 c.c. i.v. 15.0 c.c. s.c. saline susp. of bacillus of 34894.	
35808	9/1/33	2.0 c.c. i.v. 10.0 c.c. s.c. saline susp. of first bleeding diph. of 35024 ...	15/2/33 died H.W.
	23/1/33	5.0 c.c. i.v. 20.0 c.c., ditto.	
35819	9/1/33	2.0 c.c. i.v. 95.0 c.c. s.c. saline susp. diph. of 34443	Do.
	23/1/33	5.0 c.c. i.v. 17.0 c.c. s.c. saline susp. diph. of 34443.	
34550	9/1/33	2.0 c.c. i.v. 20.0 c.c. s.c. of 10 days (37° C.) K. culture of bacillus of 34894	Do.
	23/1/33	5.0 c.c. i.v. 25.0 c.c. s.c., ditto.	
	1/2/33	5.0 c.c. i.v. 35.0 c.c. s.c., ditto.	
34480	3/1/33	10.0 c.c. s.c. saline susp. of diph. of second bleeding of 34054	15/2/33 died H.W.
	23/1/33	25.0 c.c. i.v., ditto.	
34576	9/1/33	2.0 c.c. i.v. 45.0 c.c. of 10 days K culture (37° C.) of diph. of 34443 ...	Do.
	23/1/33	5.0 c.c. i.v. 27.0 c.c. s.c. of 10 days K culture (37° C.) of diph. of 34443.	
34891	9/1/33	2.0 c.c. i.v. 20.0 c.c. s.c. 10 days (37° C.) K culture of diph. of first bleeding of 35014	Do.
	23/1/33	5.0 c.c. i.v. 25.0 c.c. s.c., ditto.	
32109	9/1/33	2.0 c.c. i.v. 15.0 c.c. s.c. of saline susp. of diph. of fifth bleeding of 35024 ...	Do.
	23/1/33	5.0 c.c. i.v. 20.0 c.c. s.c., ditto.	
34390	9/1/33	2.0 c.c. i.v. 25.0 c.c. s.c. 10 days (37° C.) K culture of diph. of fifth bleeding of 35024	Do.
	23/1/33	5.0 c.c. i.v. 20 c.c. s.c., ditto.	

Sheep.		*Treatment.*	*Immunity Test.*
34420	9/1/33	2.0 c.c. i.v. 15.0 c.c. s.c. saline susp. of diph. of 34910	Do.
	23/1/33	5.0 c.c. i.v. 15.0 c.c. s.c., ditto.	
35001	3/1/33	10.0 c.c. s.c. saline susp. of diph. of 34054	Do.
	23/1/33	20.0 c.c. s.c., ditto.	
33577	9/1/33	10.0 c.c. i.v. 125.0 c.c. s.c. of 10 days (37° C.) K culture of diph. of 34054	Do.
	23/1/33	5.0 c.c. i.v. 15.0 c.c. s.c., ditto.	
34871	28/12/32	33.0 c.c. i.v. 4 days (37° C.) K.B. culture of diph. of 34894	26/1/33 died H.W.
34281	12/12/32	25.0 c.c. i.v. 6 days (30° C.) K culture of diph. of 34910	4/1/33 died H.W.
35031	29/11/32	17.0 c.c. i.v. 4 days (37° C.) K culture diph. of 34054	20/12/32 died H.W.
34894	9/11/32	90 c.c. i.v. 24 hours K culture of dipth. 34443	29/11/32 reacted. H.W. recovered.
34290	16/11/32	33.0 c.c. i.v. 24 hours K culture of diph. of 34054	20/12/32 died H.W.
35029	16/11/32	35.0 c.c. i.v. of 24 hours K culture diph. of 34054	20/12/32 died H.W.
35006	26/11/32	20.0 c.c. i.v. saline susp. of tetrad of 34910	29/12/32 died H.W.
35034	5/12/32	25.0 c.c. i.v. 4 days (30° C.) K culture of diph. of 34443	29/12/32 died H.W.
35026	12/12/32	25.0 c.c. i.v. 6 days (30° C.) K culture of tetrad of 34910	4/1/33 died H.W.

i.v. =intravenous injection.
s.c. =subcutaneous injection.
susp. =suspension.
diph. =diphtheroid.
pos. =positive.

NOTE.—Where repeated injections were given, fresh saline suspensions or fresh K cultures were injected. The saline suspensions were prepared by washing off the growths from the surface of agar (or " flooded " agar) after 24 to 48 hours' incubation. The immunity tests were arranged so that the routine virus-passage sheep served as controls. All of these died of or reacted with heartwater, but owing to the negative nature of the experiment have not been included in the appendix.