Campbell & Turner 1936
A. T. Dunn

COMMONWEALTH OF AUSTRALIA

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BULLETIN No. 97

Studies on Contagious Pleuro-Pneumonia of Cattle

II. A Complement-fixation Reaction for the Diagnosis of Contagious Bovine Pleuro-pneumonia

By

A. D. CAMPBELL, B.V.Sc. and A. W. TURNER, D.Sc., D.V.Sc.

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By

H. R. SEDDON, D.V.Sc.

II (b). The Complement-fixation Test for Pleuro-pneumonia

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A. D. CAMPBELL, B.V.Sc.

MELBOURNE, 1936

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FOREWORD.

The work which is discussed in this Bulletin forms a part of a programme of investigation which was originally put in hand as a co-operative enterprise of the former Empire Marketing Board, the Queensland Department of Agriculture and Stock, the Queensland Council of Agriculture, the cattle-owners of Queensland, and the Council for Scientific and Industrial Research.

A full account of this co-operative scheme and of the programme of investigations appeared on page 131 of the May, 1932, issue of the *Journal of the Council for Scientific and Industrial Research.*

Briefly, the Queensland interests mentioned are already finding upwards of £3,000 per annum to match a similar offer formerly made by the Empire Marketing Board through the Council, and since the 1st July, 1934, provided by the Commonwealth of Australia through the Council. The Board offered up to £5,000 per annum on a £1 for £1 basis, over a period of five years from September, 1931, but by arrangement this commitment was taken over by the Commonwealth in July, 1934.

The investigations are centred at the Animal Health Research Station, Oonoonba, near Townsville, Queensland, which was made available by the Queensland Department of Agriculture and Stock, and which has recently been modified to suit the research work under the new co-operative scheme.

A small Advisory Committee has been set up to advise generally in regard to the conduct of the researches. This Committee is now constituted as follows:—

Representatives of the State Government.—E. Graham, Esq., Under-Secretary, Queensland Department of Agriculture and Stock; A. H. Cory, Esq., M.R.C.V.S., Chief Inspector of Stock.

Representatives of the United Graziers' Association of Queensland.—E. W. Archer, Esq., F. M. Bell, Esq., Norman Bourke, Esq. (Deputy Chairman), P. A. Brown, Esq., R. C. Philp, Esq., E. E. D. White, Esq. (Chairman), J. L. Wilson, Esq.

Representative of the Council of Agriculture.—J. L. Wilson, Esq.

Representative of the Council for Scientific and Industrial Research.—Professor H. C. Richards, Chairman of its Queensland State Committee (*ex officio* member).

Dr. H. R. Seddon, Director of the Glenfield Research Station of the New South Wales Department of Agriculture, and Dr. H. E. Albiston, Director of the Veterinary Research Institute of the University of Melbourne, both of whom have experimented with the test discussed in the Bulletin, have been good enough to furnish brief accounts of their experiences and to allow them to be published.

To all who have afforded the helpful co-operation mentioned, the thanks of the Council are gratefully tendered.

PREFACE.

In this Bulletin are published the second and third papers in the serial record of the work on contagious pleuro-pneumonia of cattle, which subject forms part of a programme of investigation at the Animal Health Research Station, Oonoonba, Townsville, Queensland. The first paper was published as Bulletin No. 93, and dealt with the study of the morphology and life cycles of the causal organism.

The present paper by Mr. A. D. Campbell and Dr. A. W. Turner deals with the development of a diagnostic test based upon the well-known phenomenon of complement fixation. Dr. H. R. Seddon, Director of the Glenfield Research Station of the New South Wales Department of Agriculture, and Dr. H. E. Albiston, Director of the Veterinary Research Institute of the University of Melbourne, both of whom have experimented with the test, have been good enough to furnish brief accounts of their experiences, which are included under IIA. and IIB. of this series of papers respectively.

The Complement-fixation Test.—For the special purpose of diagnosis of this specific disease in cattle, Mr. A. D. Campbell and Dr. A. W. Turner have succeeded in adapting the technique of complement-fixation, the best known application of which is the Wasserman blood test for syphilis. The complement-fixation test is a valuable aid to diagnosis, and sometimes offers the only means of diagnosing a bacterial disease.

Contagious pleuro-pneumonia of cattle is a characteristic disease which can be diagnosed with comparative ease when it produces symptoms, but in some cases few or no symptoms are produced, and diagnosis is quite impossible by ordinary inspection and even closer examination. Some cattle appear to recover, but may still have affected areas in their lungs, and may spread the disease among susceptible cattle. The slaughter of all apparently affected cattle usually fails to eliminate infection from the herd because the apparently recovered cases and those in the early stages of the disease are left to spread it. The eradication of contagious pleuro-pneumonia from the herd, therefore, depends upon the accurate diagnosis of all animals harbouring the infection. The complement-fixation test as elaborated by Campbell and Turner is capable of detecting these animals, and so makes possible, without slaughter of every animal, eradication of the disease from those of our herds which are small enough for complete mustering and individual identification of animals. It can have little application in the larger herds in the unfenced country, except for the purpose of detecting infection in individual animals mustered and held preliminary to travelling. If infected animals are eliminated from such "mobs," they can be travelled in the security that pleuro-pneumonia will not occur and cause serious losses on the road, or the spread of the disease in "clean" areas.

The use of the test in the field and under experimental conditions has increased our knowledge of the disease considerably. With its aid, animals in the early stage of infection have been detected, whereas several weeks have elapsed before such animals have shown definite clinical symptoms, all the while being a potential danger to healthy in-contact animals. The test has enabled us to determine that the development of definite symptoms may, on occasions, be slow or even absent, and with this added knowledge control measures can be made more effective.

The test is extremely specific, possibly more specific and more efficient than any similar test used as an aid to diagnosis of disease in man or animals. In spite of this, it cannot be undertaken lightly. Its conduct involves great concentration by a skilled and experienced worker. It cannot be undertaken in a field laboratory, but this offers little or no difficulty to its practical application, as specimens of blood or serum to be tested can be preserved and transported over long distances without deterioration. The test has already had extended trial, and the papers by Dr. Seddon and Dr. Albiston bear witness to the fact that it has proved highly reliable in hands other than those of Campbell and Turner. In order that it may be applied in all parts of the Commonwealth at any time, some modification in the preparation of the antigen is necessary. Progress is already being made in this direction.

The Distribution of the Organism in the Animal Body.—The third study of this series has been made in order that we should be better informed on the capacity of the organism to invade the tissues of the animal body, and on the extent of the invasion. As the main lesion produced in infected cattle is a pneumonia, it is natural that attention has been focussed on this lesion, and that many workers have assumed the localization of the causal organism in the lung lesions only. The results of the present study add to our knowledge of the disease, and confirm and extend the observations made more recently by workers in other parts of the world.

With this added knowledge, the experimental reproduction of the disease has been made possible, which enables an accurate study of resistance to infection following vaccination to be undertaken. The results of these further studies will be published in future papers.

L. B. BULL,
Chief, Divisions of Animal Health
and Animal Nutrition.

314 Albert-street, East Melbourne.
November, 1935.

SUMMARY.

II.—A Complement-fixation Reaction for the Diagnosis of Contagious Bovine Pleuro-pneumonia.

1. A complement-fixation test for the diagnosis of contagious bovine pleuro-pneumonia, in which Ebert and Peretz's antigen is used, is described. The technique is based upon orthodox Wassermann procedures suitably modified, and permits the performance of the test with accuracy and reasonable speed. There is a complete absence of "fleeting reactions."

2. The test is extremely specific. Sera from 2,059 Tasmanian cattle known to be free from pleuro-pneumonia gave completely negative reactions, and another three gave only a faint trace of fixation ("one plus") in a one-in-ten dilution; a large variety of pathological conditions was represented among 422 of them.

3. The complement-fixation responses to various methods of inoculation with pleuritic exudate and cultures are discussed, and the value of the reaction in experimental investigations is indicated.

Cattle vaccinated at the tip of the tail by the Willemsian method rapidly develop complement-fixing antibody in the blood stream from two to eleven days later. The titre may reach 1 in 200 (or even higher). The maximum occurs from the 5th to the 22nd day, and the titre then begins to fall rapidly. The longest period during which an animal showed complement-fixing antibody in the blood stream following uncomplicated tail-vaccination was 37 days. It is recommended that an interval of eight weeks should be allowed after uncomplicated tail-vaccination, before cattle are submitted to the test for diagnostic purposes.

4. The complement-fixation response to natural infection is discussed. In the elaboration of this pleuro-pneumonia test as applied to the diagnosis of the disease, an entirely novel procedure was adopted, viz., the establishment of a naturally-infected herd containing susceptible and insusceptible cattle, and the regular testing of their sera at weekly intervals over a period of two years, combined with clinical and post-mortem examinations. By this means, complete serological and clinical histories of most of the cattle concerned were obtained, embracing the pre-infectional, infectional, and post-infectional phases, and it is consequently believed that the results obtained and the conclusions reached have a special significance. Additional information was gathered at abattoirs.

A positive reaction may often be obtained before the manifestation of clinical symptoms. As with tail- and subcutaneously-vaccinated animals, complement-fixing antibody usually increases very rapidly in

the circulating blood once infection occurs; the titre may reach 1 in 640 in acute cases. Cases in which the causal organism was confined to the mediastinal lymphatic glands have been detected. Cattle, apparently recovered but remaining carriers of viable organisms in encapsulated pulmonary lesions, usually give persistent positive reactions. The sera of recovered animals showing, *post mortem,* only old fibrous pleural adhesions do not usually contain detectable complement-fixing antibody.

Twenty-five out of 25 cases of acute pleuro-pneumonia (i.e., 100 per cent.) were detected by means of this test. Forty-two out of 45 chronic cases with sequestrum formation (i.e., 93.3 per cent $\pm$ 3.65 per cent.) gave positive results, and the correlation might be higher, for, owing to the impracticability of bacteriological confirmation in all cases, the possibility that the remaining three were "sterile" cases in which the infection had died out cannot be excluded.

It was definitely shown that cattle can recover almost completely from pleuro-pneumonia to an extent that makes the recognition of their earlier infection very difficult. Another important finding was that a certain number of animals may contract acute pleuro-pneumonia of such a mild degree as to be sub-clinical and detectable only by serological means.

5. Measures for the use of the complement-fixation test in the control of contagious bovine pleuro-pneumonia under various conditions in Australia are discussed. These include dairy herds in the closely-settled districts, cattle-breeding or fattening properties of moderate size, and large mobs travelling by road from the enormous pastoral properties to the markets and abattoirs.

6. It is expected that the test will greatly facilitate the control of outbreaks of the disease, and will permit the safe transfer of cattle from infected to uninfected areas.

II (a).—Observations on the Diagnosis of Bovine Contagious Pleuro-pneumonia by Means of the Complement-fixation Test of Campbell and Turner.

This paper records the results obtained at the Glenfield Veterinary Research Station in the application of the test. The latter was found to possess a very high degree of reliability, animals known to be free from contagious pleuro-pneumonia giving negative reactions, whereas all known affected animals reacted. Moreover, by its use a number of non-clinical cases were detected, and the value of its application in the control of this disease under field conditions has been demonstrated in the case of one large herd.

II (b).—The Complement-fixation Test for Pleuro-pneumonia.

In this paper, the results of the application of the Campbell and Turner test to some 800 bullocks brought into Victoria from a property where pleuro-pneumonia had occurred are discussed. All the positive reactors were confirmed at autopsy, and the continued freedom from pleuro-pneumonia of the survivors for the subsequent 27 months is taken as evidence that all infected animals were detected.

III.—A Cultural Study of the Distribution of the Specific Organism *Borrelomyces peripneumoniae*, throughout the Body of Animals Naturally and Artificially Infected.

The causal organism of contagious pleuro-pneumonia was usually found in the blood stream in acute cases; but in chronic cases with "encapsulated" or "sequestrated" lung lesions it was usually recovered only from the lung lesion, though occasionally, also from the mediastinal and bronchial lymph glands.

It was found in the blood stream early in the infection, but its presence there subsequently was intermittent. Blood culture tests are therefore of little practical diagnostic value.

It was found to be constantly present in the pleuritic exudate, the specific lung lesion, and the mediastinal and bronchial lymph glands of active cases. It was also found to exist in many other parts of the body without causing obvious lesions.

The specific organism could never be cultivated from completely recovered cases where the only remaining lesions were local pulmonary fibrosis or chronic pleural adhesions.

It was cultivated from organs of some foetuses following artificial infection of the mother.

Smaller laboratory animals (sheep, goats, and rabbits) were artificially infected, and the organism was recovered from various parts of the body.

Studies in Contagious Pleuro-Pneumonia of Cattle.

II. A Complement-fixation Reaction for the Diagnosis of Contagious Bovine Pleuro-pneumonia.

Its Use in Experimental Investigations and in the Control of the Disease.

By A. D. Campbell, B.V.Sc., and A. W. Turner, D.Sc., D.V.Sc.†*

I. INTRODUCTION.

Pleuro-pneumonia of cattle, since its introduction into Victoria in 1858, has spread to every State on the Australian mainland, and has caused enormous losses, both directly and indirectly.

The Chief Inspector of Stock for New South Wales, Colonel Max Henry, in his Presidental Address to Section L (Veterinary Science) of the Australasian Association for the Advancement of Science in 1928, gave the following statement of its extension at that time:—

"In Queensland it is reported occasionally from the coastal areas, but the parts most seriously affected are the west and north-west, areas of large runs on which control is very difficult. New South Wales is constantly being reinfested from Queensland, and in its turn reinfests Victoria. The coastal areas of New South Wales, in which are the great dairying districts, are remarkably free. Victoria presents a different picture to any of the other States, and appears to be the State in which the disease has the most consistent grip.‡ Here it is not a disease of the big runs but of closely settled dairying and agricultural country. South Australia and the south-western portion of Western Australia appear to be very free, the disease being practically only seen in cattle brought down from the north. In the north-west of Western Australia and in Northern and Central Australia the situation is obscure, but the disease is certainly present in the two first-mentioned areas."

In the early decades that followed its introduction, when it apparently ran a hyper-acute, fulminating course through a highly susceptible bovine population, it caused enormous mortalities. Although such catastrophic direct losses are rarely seen now, yet it has other serious aspects, including interference with overseas export, interstate trade, and transfer of cattle within the State. In Queensland, its importance becomes aggravated during the travelling of stock to markets, when,

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† Officer-in charge of the Council's Animal Health Research Station, Oonoonba, near Townsville, Queensland

‡ This statement is no longer correct: the Department of Agriculture, with the aid of the test described herein, speedily controls the occasional outbreaks and at the present time the State is free of the disease

owing to the outbreak of pleuro-pneumonia, mobs may be detained in quarantine for long periods. It costs approximately £10 per day to detain a mob of 1,000 cattle on the road, and, as the usual quarantine period is 60 days after the appearance of the last case, the monetary loss is very great; furthermore, the opportunity for sale has then often been lost.

The important problem of pleuro-pneumonia is the detection of the "carriers," i.e., the apparently healthy animals with specifically infected pulmonary sequestra. It is these animals that introduce the infection into susceptible herds. There is no known clinical method of detecting these sources of contagion, but research work has indicated the possibility of revealing them by serological reactions.

Henry (l.c.) has declared that "the two main lines along which research may be expected to come to the aid of the authorities concerned in the control of the disease are the elucidation of a method of diagnosis apart from clinical observation or post-mortem demonstration, and improvement in methods of protective inoculation which will do away with the fear of untoward sequelae."

It has been the object of the investigation described herein to devise a reliable practicable complement-fixation reaction and so to place in the hands of Government Stock Departments an essential weapon in the campaign against pleuro-pneumonia, to wit, the ability to detect the "carriers" of the disease.

II. HISTORICAL.

The first attempt to use the complement-fixation reaction in the diagnosis of pleuro-pneumonia was made in Russia by Schochowsky (1912) who, using as antigens extracts from affected lungs, inflammatory exudate, and cultures, tested sera from natural causes of pleuro-pneumonia as well as experimentally infected and immunized calves. He decided that the results were not sufficiently accurate. In Germany, Poppe (1913) used as antigens the subcutaneous inflammatory swellings of artificially infected calves, similar material dried and powdered, lung tissue, pleuritic exudate, and cultures; but both he and Meyer (1914) substantially agreed with Schochowsky.

Following its introduction from Poland into Germany during the War, research on its serological diagnosis received a great impetus. Titze and Giese (1919) found saline extracts too weak, but boiled extracts and alcoholic extracts of infected lung tissue or bronchial glands gave good results; they were troubled, however, with the so-called "fleeting reactions," i.e., the previously intact erythrocytes in certain tubes, indicating fixation of complement, would suddenly become lysed, the sera thereby behaving as pleuro-pneumonia negatives. Miessner and Albrecht (1920) and Lensch (1921) also complained of *"fleeting reactions" and the consequent difficulty of reading the test.* In 1921, Giese claimed good results with antigens made from old cultures in horse-serum Martin's broth or horse-serum ox-meat infusion. In 1923, Titze, Giese, and Wedemann published a monograph on pleuro-pneumonia in which further work on their complement-fixation test was reported; Giese's antigen was used, and the complement was titrated in two series in the presence of positive and negative sera

respectively, that dose being selected which gave the sharpest differentiation between positive and negative sera. The time for reading results varied with the complement and had to be very carefully determined, and the reactions read at the moment the negative-control tubes become completely haemolysed; beyond this time, there was danger of "fleeting reactions." They claimed excellent results with their test, and a further report was published by Giese and Wedemann in 1924. However, Dahmen (1923-24), not being satisfied with results obtained with Titze and Giese's antigen, modified it by using as antigens alcoholic and carbol-saline extracts of the centrifugates of young glucose serum broth cultures. Even so, his results do not appear to be strikingly good. Karmann and Witte (1926) applied Titze and Giese's antigen and general procedure to a test using active serum, and claimed to detect a larger number of positives than by using inactivated sera. Ziegler (1927) carried out exhaustive researches with a modification of Titze and Giese's method, and correlated the reactions with the age and extent of the lesions; he reported not having encountered "fleeting reactions," and claimed very good results, i.e., 93 per cent. of acute cases and 83 per cent. of cases with more or less large sequestra; but his test differed slightly from that of Titze and Giese's in the use of an excess of complement over that specified by their method.

Dzius (1925), working in Poland, claimed good results from the complement-fixation test; but no information on the antigen or technique is available.

Walker (1923), in Kenya Colony, claimed to have obtained "practical results" (*sic*), and that the fleeting reactions which obscured the results in the complement-fixation test were eliminated by using the conglutination method as indicator.

Japanese workers have also interested themselves in complement-fixation. Nakamura, Futamura, and Watanuki (1926) investigated the complement-fixation test, among other serological reactions, using as antigen saline emulsions of colonies from the surface of horse-serum agar slopes incubated for ten days; the emulsions were heated at 60° C. for three hours and 0.5 per cent. of phenol was added. They claimed it was superior to all other antigens tested. The performance of the test apparently followed orthodox methods of titrating complement, &c., and gave more satisfactory results. "Fleeting reactions" are not mentioned. In a subsequent publication, Futamura and Watanuki (1927), after examining antigens prepared in 13 different ways, concluded that the most potent was a saline emulsion of colonies (heated to 60° for one hour); Giese's culture antigen was also found to be of value. Kabashi, Yamagiwa, and Ito (1930) investigated the correlation between degree of fixation and age of lesions; they claimed good results, but did not describe the technique or antigen.

In Australia, the first work on complement fixation in pleuropneumonia was performed by Heslop (1921, 1922), who finally selected as antigen alcoholic extracts of dried subcutaneous oedematous tissue, with which he obtained fairly good results. However, the reading of the test required constant supervision on account of "fleeting reactions", and he abandoned it in favour of agglutination, considering that its

technique was "too intricate and laborious to allow of its adoption as a routine diagnostic method." In 1925, one of us (A.W.T.) took up the study of Titze and Giese's technique; the usual trouble with "fleeting reactions" was encountered, and frequently great difficulty was experienced in reading the reactions. For unavoidable reasons, the work was not carried very far, though the conclusion was reached that the test had a certain value, but that it should be limited to sera taken from animals before prophylactic vaccination had been carried out. Unfortunately, the work had to be abandoned at this stage, but it was carried on and extended by Gregory (1927), who reported that the test was "worthy of use under field conditions". He also complained of the "fleeting reactions", and reported that prophylactic tail-inoculation was followed by a positive complement-fixation reaction. In New South Wales, the same method was applied by Hindmarsh (1933), who claimed that cultures 14–21 days old yield antigens as satisfactory as the 6–8 week cultures; he did not find the time factor of such importance, and was apparently not troubled with "fleeting reactions". He reported very good results. Ebert and Peretz (1928), who introduced the boiled antigen used by us, reported good results from its use in a complement-fixation test, but their communication is apparently only of a preliminary nature.

A critical reading of the published work and our own earlier experience convinced us that the only techniques of which full details were available were too cumbersome and too subject to "fleeting reactions" (and consequently to false-negative reactions), that many reports were conflicting, and that, therefore, independent investigations were necessary rather than the adoption of any of the suggested methods.

Expressions such as "good results", "practical results", and "excellent results" abound in the literature, but very rarely is statistical support offered for the claims made. No systematic investigations appear to have been made into the serological response of animals to the Willemsian prophylactic vaccination, which is widely practised in Australia, nor to its influence upon the interpretation of the reaction; nor has any attempt been made to trace the rise and fall of specific circulating antibody in cattle from before their infection with pleuro-pneumonia until their eventual death or recovery. In previous investigations it has been usual to base the reports upon tests conducted upon cattle concerned in natural, fortuitous outbreaks of the disease. Nothing was known of their earlier serological history, and, as a matter of disease control, they were usually killed out, both clinically affected and apparently healthy, and the observations thereby concluded. In the present investigation a new procedure was adopted, namely, the continued serological examination of cattle deliberately exposed to infection and kept under observation for as long as two years. Consequently, we believe that the results obtained and the conclusions reached have a special significance, and that the reaction, when carried out by the technique described, has a reliability, both in experimental investigations and in the control of the disease, that makes it of very great value.

For the sake of clarity, the technique of the test will first be described and then the experimental work on which its interpretation was based.

III. TECHNIQUE OF THE REACTION.

The essentials for a complement-fixation test as a diagnostic procedure, especially with bovine sera, are as follows:—

(*a*) The antigen must be stable, easily prepared, and not unduly expensive.

(*b*) The technique must be as simple as possible, and readily capable of standardization.

(*c*) The test must be sensitive, i.e., capable of demonstrating the presence of very small amounts of antibody, and it must be highly specific.

(*d*) The reaction must be easy to read, and there must be a complete absence of "fleeting reactions".

(*e*) It must be capable of being carried out with reasonable speed.

We believe that the test described in this Bulletin fulfils the above conditions to a very high degree: the antigen used is one described by Ebert and Peretz (1928); the technique is based upon orthodox Wassermann procedures, but is modified for the above purposes; the test is extremely sensitive and specific; and there is a complete absence of "fleeting reactions", which are usually so troublesome with bovine sera. All reagents are adjusted so that the required amounts are contained in the one unit volume of 0.5 ml.*, and all additions of reagents are performed by means of rubber-teated pipettes or Vernes' rhéomètres; by this means the technique is enormously simplified, and much greater speed combined with accuracy is possible.

1. Special Apparatus.

(*a*) A hemicylindrical water-bath of 8 gallons capacity, holding ten removable wire racks each capable of holding four rows of twelve tubes, and fitted with a sleeve to hold a Roux bimetallic gas thermo-regulator 1 metre long, adjusted to maintain a constant temperature of 37.5° C.

(*b*) A water-bath regulated at 56° C. for the inactivation of test sera.

(*c*) Test tubes of clear glass with walls 1 mm. thick, 7.5 cm. long, and with an internal diameter of 9mm.

(*d*) Accurately graduated pipettes of 10, 5, and 1 ml. capacity, respectively.

(*e*) One shortened 1 ml. pipette graduated to read 0.25, 0.5, and 0.75, and 1.0 ml., and furnished with a rubber teat.

(*f*) One 100 ml. graduated measuring cylinder, several beakers, and 250 ml. Erlenmeyer flasks.

(*g*) Suitable ampoules, bottles, or test tubes for the collection of blood.

2. Glass Cleaning.

Reagents should not be allowed to dry on the glassware, as efficient cleaning is thereby made difficult. After a wash with tap water, the pipettes, test tubes, and Wassermann tubes are placed for twelve hours in a solution made by dissolving in one gallon of rain water,‡ 1 oz. of a

* The use of "ml." instead of "cc." is followed throughout the report.

‡ Where tap water is suitable it may be used; at this laboratory it is too "hard."

mixture consisting of 40 per cent. commercial trisodium phosphate, and 60 per cent. commercial sodium carbonate. They are then removed, rinsed several times in rain water, dried in a hot-air oven, and kept protected from dust and other extraneous matter.†

3. Reagents.

(*a*) Serum to be tested.

(*b*) Complement (guinea-pig serum).

(*c*) Antigen.

(*d*) Sheep's red blood corpuscles.

(*e*) Anti-sheep haemolytic amboceptor (rabbit).

(*f*) Normal saline (0.85 per cent. of sodium chloride (B.D.H., A.R.) in distilled water). For the performance of the test, we have not found it necessary to use saline prepared from glass-distilled water, but we prefer it in the preparation of antigen.

4. Serum.

(i) *Collection.*

(*a*) *Jugular or Caudal Blood.*—3–5ml. of blood are taken from one of the jugular or caudal veins into sterile test tubes, &c., by means of stout hypodermic needles or narrow canulas, but in case of necessity it may be collected from the tail merely by cutting a vein. The receptacles are then laid at an angle of approximately 15° with the horizontal to give a long sloped surface, by which means a good exudation of serum is generally produced. This is usually removed on the day of collection, although it is apparently of no disadvantage, in the absence of bacterial contamination, to leave it on the clot for several days. Sera should be stored at low temperature in a refrigerator. After standing thus overnight, they become quite clear by sedimentation; if they are to be tested on the day of the bleeding, it is nearly always necessary to centrifuge them. Although the stroma of lysed red blood corpuscles is commonly regarded as highly anti-complementary, we find that a slight degree of haemolysis in the sera has no ill effect.

(*b*) *Heart Blood Collected Post Mortem.*—When it is not possible to collect jugular or caudal blood as described above, as during post-mortem examination at abattoirs, it may be obtained immediately after removal of the thoracic organs by incising the right ventricle of the heart and allowing the blood to run into a test tube. The subsequent operations are as above. With this method of collection, it has been noticed that a secondary clotting of the removed serum occurs much more frequently than when blood is collected *ante mortem*, and it is then necessary to cut up the delicate gelatinous mass and centrifuge the serum from it.

(ii) *Preservation of Sera for Transport.*

It is often not practicable, under field conditions, to collect sera aseptically; hence, in order to suppress bacterial growth, we have examined the possibility of adding antiseptics to them before transport to the laboratory. The following procedure has been found satisfactory. Blood is collected by any of the above methods, allowed to clot, the serum decanted, and, if necessary, blood cells are separated by sedi-

† As particles of tobacco ash are said to exert a marked anti-complementary effect (Wyler, 1929), smoking during the progress of the test is to be avoided.

mentation; two drops of chloroform are added to the clear serum, and then the bottle is well stoppered, preferably with cork. Using this technique, sera have been sent in the post from Perth to Townsville, a distance of 3,000 miles occupying ten days in transit, with a complete absence of contamination, no appearance of anti-complementary properties, and apparently no destruction of antibody. On receipt, such sera may present a milky, unattractive appearance due to partly emulsified chloroform, but, on standing in the refrigerator overnight, a complete separation occurs, and the clear serum may be pipetted from the chloroform, which will have sunk to the bottom.

In a few cases, toluol has been used to preserve sera sent from Melbourne to Townsville, a distance of 1,800 miles occupying five days in transit, with no apparent ill effects. The addition of 0.25 per cent. of carbolic acid likewise has caused no loss of titre or the development of anti-complementary properties in high-titre positive sera at room temperature over a period of three months.

(iii) *Inactivation.*

On the day of the test, sufficient of the clear serum for the test is diluted 1 in 10 with normal saline and inactivated *in the diluted state* at 56° C. for 30 minutes. During this operation, slight traces of chloroform in preserved samples are apparently removed.

5. Complement.

The fresh sera of a sufficient number of healthy male guinea pigs weighing over 500 grams are used. The blood, obtained by cutting the throat after stunning, is allowed to clot in sterile petri dishes, the clot cut up with a scalpel, and placed in the incubator at 37° C. for one half hour and in a refrigerator for a further hour. The serum is then pipetted off into centrifuge tubes and clarified by centrifuging at 3,500 revolutions for five minutes. When not actually in use, it is kept in the refrigerator. Excess complement is stored by freezing and is suitable for use for four days after collection.

6. Antigen.

This is prepared according to the technique described by Ebert and Peretz (1928) by extracting, in distilled water at boiling point, either fresh pleuritic exudate coming from natural cases of pleuro-pneumonia or the subcutaneous inflammatory exudate that results from the subcutaneous inoculation of infective material. It has been our experience that antigens prepared from the former have a higher antigenic value than those prepared from the latter, and we therefore prefer to use pleuritic exudate. Boiled antigens or "Kochextrakte" have been used by earlier workers; thus Titze and Giese (1919) used among other antigens "Kochextrakte" prepared by boiling fresh, minced, infected lung or bronchial glands in distilled water for 30 minutes, clarifying by centrifugation, and making isotonic with sodium chloride; very good results were reported but "fleeting reactions" were encountered. Walker (1923) used similarly prepared extracts made from subcutaneous oedematous tissue for the conglutinin reaction, but found them too anti-complementary for use. Futamura and Watanuki (1927)

used both Titze and Giese's "Kochextrakte," which were too anti-complementary in the doses used, and boiled extracts of colonies on serum agar, which were inferior to similar antigens heated to 60° C. for one hour instead of to boiling point. There has been a general tendency, within recent years, for the use of culture antigens rather than tissue antigens.

Preparation.—A measured volume of pleuritic exudate diluted by the addition of four volumes of glass-distilled water is placed in an Erlenmeyer flask; the contents are raised to boiling point by placing the flask in a boiling brine-bath and are kept gently boiling for ten minutes, during which coagulation occurs. Excessive frothing is controlled by blowing air over the boiling mixture through a bent glass tube. The mixture is then filtered through Whatman No. 1 paper to remove coagulum, and the volume of filtrate measured. To make the extract isotonic, chemically pure sodium chloride (B.D.H., A.R.) is added at the rate of 0.85 gms. per 100 ml. of filtrate after subtracting the original volume of exudate used. Phenol is then added at the rate of 0.25 gm. per 100 ml. of filtrate and dissolved by shaking. The mixture is then re-filtered to remove a slight precipitate that results. This fluid, known as antigen, is stored in glass-stoppered bottles in a refrigerator. It is now ready to be tested for sensitivity in parallel with the antigen already in use. Antigens prepared as above have proved almost uniformly satisfactory and are very stable, no loss of titre being apparent during at least twelve months.

7. Haemolytic System.

(i) *Sheep Red Cells.*—Blood is collected aseptically by jugular puncture from a suitable and satisfactory sheep into an equal volume of 2 per cent. sodium citrate solution in normal saline. The mixture is centrifuged, and the cells washed three or four times with normal saline. If they show signs of fragility (haemolysis) on washing, they are discarded and another sheep selected. The citrated, unwashed blood may be kept at a temperature of from 3° to 4° C. for a week.

(ii) *Haemolytic Amboceptor.*—This is prepared in rabbits by means of sheep red cells, and is either purchased from the Commonwealth Serum Laboratories or made by any of the well-known methods; it is preserved by the addition of 50 per cent. by volume of glycerine and stored in the cold. Amboceptor preserved by the addition of 1:20,000 of mercuric chloride has also proved satisfactory.

(iii) *Sensitization.*—For use, the washed sheep red cells are packed by centrifuging at 3,500 revolutions per minute for 20 minutes. The supernatant saline is removed and the packed cells drawn into a pipette. A suspension of 6 per cent. by volume is made in normal saline. To the required volume of this suspension is added an equal volume of normal saline containing amboceptor at the rate of 6 minimum haemolytic doses (M.H.D.) per volume (0.5 ml.); by this means a 3 per cent. suspension of red cells in normal saline containing 3 M.H.D. per unit volume is obtained. Sensitization at room temperature for 1 hour is allowed. No further washing or removal of excess amboceptor is carried out.

8. Titration of Haemolytic Amboceptor.

Certain precautions are necessary in titrating an amboceptor.

(1) Complement should fulfil the requirements as set out under "Titration of Complement" (i.e., sufficient titre and absence of natural anti-sheep haemolysins).

(2) Red cells should be unpreserved and otherwise suitable.

(3) As a check on reagents employed, an amboceptor should be titrated in parallel with a tested amboceptor of known titre.

A rack is taken containing three rows of test tubes. In the first row, an accurate series of saline dilutions of amboceptor is prepared thus: 1 in 10, 50, 100, 500, 1,000, 2,000, 3,000, 4,000, 5,000 or even further if necessary. To the second row is added one unit volume (i.e., 0.5 ml.) of the double strength (i.e., 6 per cent.) suspension of unsensitized red blood cells, using the rubber-teated pipette, and taking care to deliver cells to the bottom of the tubes. Commencing with the tube containing the highest dilution, one volume is taken from the first row, which contains the amboceptor dilutions, and is added to the corresponding tube immediately behind it, each tube being individually shaken from side to side. Each tube in the second row now holds two volumes (i.e., 1.0 ml.) containing 3 per cent. of sheep red cells and a dilution of amboceptor twice that added, i.e., the series now runs 1 in 20, 100, 200, 1,000, 2,000, 4,000, 6,000 8,000, 10,000, &c. The rack is covered with a clean, smooth towel, and left at room temperature for one hour to allow sensitization, the whole rack being shaken vigorously from side to side every fifteen minutes. The first three tubes containing the dilutions 1:20, 1:100, and 1:200, respectively, are removed and left for a further hour and a half at room temperature. They are then gently shaken and examined with a hand lens for signs of haemagglutination: if this occurs in a dilution as far as 1:100, it will be necessary during the use of that amboceptor to shake sensitized cells very thoroughly before use; if it extends beyond that dilution, haemagglutinins should either be removed by absorption and the amboceptor re-titrated, or the sample should be rejected.

Now, starting from the highest dilution at the right, one volume of the sensitized cells from each tube of the second row is pipetted to the bottom of the empty tube immediately behind it, after which two volumes of normal saline and one volume of complement dilution containing 2½ M.H.D. (see "Titration of Complement") are added, making a total of four volumes in each tube, as is used in the test proper. A tube containing one volume of 3 per cent. R.B.C. and three volumes of normal saline should be included to control the fragility of the cells used; if any trace of haemolysis occurs they must be rejected.

Each tube is shaken to mix the contents, and the rack placed in the water-bath at 37.5°, removed every 10 minutes and well shaken, and after a total of 30 minutes removed for reading. The highest dilution of amboceptor from the second row producing complete haemolysis contains one minimum haemolytic dose per volume. If this dilution is less than 1 :1,000, the amboceptor is not satisfactory, being apt to give obscure results in the test proper.

Amboceptor, being very stable, need only be titrated once every three months.

9. Titration of Complement.

On the day of the test, this is the first operation carried out, in order that the least possible delay between it and the test proper may occur. Make a 1 in 10 normal-saline dilution of guinea-pig serum by adding one volume (0.5 ml.) to nine volumes (4.5 ml.) of saline, and using the same teated 1.0 ml. pipette* as is used in the test proper. Arrange nine tubes in a row; to these add, from left to right, 1, 2, 3, ... up to nine volumes of saline; and then add to each tube one volume of the 1:10 complement dilution, giving a series of complement dilutions from 1 : 20 to 1 : 100. Two rows each of nine tubes are now taken. The tubes in the first row will eventually contain two volumes of saline, one volume of the appropriate complement dilution, and one volume of sensitized red cells; those in the second row, one volume of appropriate complement dilution, one volume of antigen, and one volume of sensitized red cells. Two additional control tubes are placed in the rack; control number 1 contains three volumes of saline and one volume of sensitized red cells, and is a cell-fragility and/or normal-saline control; control number 2 contains two volumes of saline, one volume of a 1:10 dilution of complement, and one volume of *unsensitized* 3 per cent. red cell suspension, and is a control for the absence of anti-sheep haemolysins in the complement.

In setting up the above rows, the saline is first delivered to all tubes; then, working from the highest to the lowest dilution, the respective complement dilutions are added.† As a precautionary measure, it is desirable at this stage to place the original series of complement dilutions in an ice-chest; they are then available without having undergone deterioration should it be necessary to repeat the titration. To each tube in the first row, add one volume of the well-shaken sensitized red-cell suspension; to the control tube No. 1, add one volume of sensitized red cell suspension, and to No. 2 one volume of 1:10 complement dilution and one volume of 3 per cent. suspension of unsensitized red cells. Shake the racks well every ten minutes. After 30 minutes, remove them from the bath and read the titre of the complement. The two control tubes must show no trace of haemolysis.

The titre is read from the tube in which lysis is almost, but not quite, complete (the tube in which there is still a slight opacity); this will be found at the right of the highest dilution of complement showing complete, sparkling haemolysis.

Remove row 2 (i.e., containing antigen) to another rack and add to each tube one volume of sensitized red cells; replace in bath, shake as before, remove after 30 minutes, and read and ascertain the titre of complement as for row 1. If the titre in row 2 does not lag more than one tube behind that in row 1‡, the titre in row 1 is accepted. Very occasionally it may happen that the titre in row 2 lags considerably more than one tube behind that in row 1; this means that the particular complement is unduly sensitive to absorption by the antigen

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* When proceeding from one reagent to another, the pipette is cleaned as follows:— A series of three beakers of saline is set up, the second one being kept near the boiling point. The pipette is first washed in beaker 1 by sucking cold saline in and out several times; the same operation is repeated then in the hot saline in beaker 2, and finally in beaker 3.

† When delivering dilutions (similarly in preparing serum dilutions in the test proper), the fluid must be pipetted to the bottom of the tubes, and drawn up and expelled several times.

‡ i.e. The antigen may absorb a small amount of complement (see next page).

and therefore must be replaced by another. If the tube containing the 1 : 50 dilution of complement shows complete lysis, then the tube containing 1 : 60 dilution will show almost, but not quite, complete lysis; therefore the titre of this hypothetical complement would be taken as 1 : 60. It is desirable that the titre fall between 1 : 40 and 1 : 80; we have found it desirable to discard those below 1 : 40.

In this test the dose of complement is 2½ M.H.D.; therefore, in the hypothetical case, a dilution of 1 : 24 with normal saline is made. The making of this dilution may be deferred until needed, or, if diluted, the complement must be stored in the ice-chest.

10. Standardization and Titration of Antigen.

Before a new antigen is accepted, it is subjected to the following tests:—

(i) Parallel titration of a known satisfactory antigen and the new one against 2½ M.H.D. of complement to test it for anti-complementary properties.

(ii) Testing of the antigen for absence of haemolytic properties.

(iii) Parallel titration of a known satisfactory antigen and the new one in graded dilutions against several known positive and negative sera.

(iv) Parallel titrations of a number of positive sera varying from very strong to very weak, and an equal number of negative sera against a known satisfactory antigen and the new one in a dose of from 5 to 10 antigen units* (A.U.).

(v) Specificity tests where possible.

(vi) Repetition of an ordinary day's test with the new antigen.

The above points are now discussed in more detail.

(i) A rack containing four rows each of twelve tubes is taken. To the tubes of rows 1 and 2, dilutions of the old and new antigens are added as follows: undiluted, 1 in 2, 1 in 3, 1 in 4, 1 in 5, 1 in 10, 1 in 15, 1 in 20, 1 in 25, 1 in 30, 1 in 35, and 1 in 40.

One unit volume, beginning at the highest dilution, is added from row 1 to the corresponding tubes in row 3, and similarly from row 2 to row 4. Rows 1 and 2 are now temporarily put aside. To the tubes in rows 3 and 4 one volume of saline is added; then one volume of saline containing 2½ M.H.D. of complement. After shaking, the rack is placed in the water bath held at 37.5°C., removed for shaking every 10 minutes, and after 30 minutes removed. One volume of sensitized red cells is then added to each tube and the tubes shaken. They are replaced in the water bath, shaken every 10 minutes, removed and read after 30 minutes. The readings of both rows should agree, i.e., the antigen must not be unduly anti-complementary. Occasionally, there may be partial absorption of complement in the tube to which undiluted antigen has been added.

(ii) During the setting-up of the previous test, an extra tube is added containing two volumes of saline, one volume of undiluted new antigen, and one volume of 3 per cent. unsensitized red cells. After

* In practice, it is found that 5 A.U. are sufficient

the results of the antigen-complement titration are obtained (i.e., one hour), this tube is removed and centrifuged; if the supernatant shows traces of haemolysis, the antigen should be discarded. In our experience this has never occurred.

(iii) A rack is taken containing 4 rows of 12 tubes. To the tubes in rows 1 and 3, one volume of a 1 in 10 dilution of an inactivated negative serum is added; to the tubes in rows 2 and 4, the same procedure is carried out with a positive serum. To each tube one volume of saline containing 2½ M.H.D. of complement is added. Using the dilutions already prepared in test 1, and commencing with the highest, one volume of the known satisfactory antigen is added to the corresponding tubes in rows 1 and 2, and one volume of the new antigen similarly to the tubes in rows 3 and 4. In practice, it is simpler in the case of the known-negative serum to limit the tubes to 6 for the lesser dilutions. The test is now carried on as for the test proper and the tubes read. In addition, a serum anti-complementary control tube for each serum must be put up. If the new antigen is to be usable, it must firstly give with the known negative serum similar results to those obtained when the known satisfactory antigen was used, i.e., no fixation or at most only a trace of fixation in the tube to which undiluted antigen was added; and, secondly, with the known-positive serum there must be complete fixation at least in the tube containing the 1 in 10 dilution of antigen.

The antigen unit (A.U.) is contained in unit volume of the highest dilution giving complete fixation with 2½ M.H.D. of complement in the presence of a strongly positive serum.

(iv) Saline dilutions of both antigens are prepared containing 5-10 A.U. per volume. A number of sera varying from very weak to very strong positives are set up in two duplicate rows in dilutions 1 in 10, 1 in 20, 1 in 40, up to 1 in 200. The test is then carried out as for the test proper, excepting that the known satisfactory antigen is added to one row and the new antigen to the duplicate row. At the same time a number of known-negative sera are similarly tested in duplicate excepting that the dilutions are only 1 in 10, 1 in 20, and 1 in 30. Occasionally, it may be found that an otherwise satisfactory antigen lacks the power of detecting very weak positive sera, so that its lack of sensitivity warrants its rejection.

(v) The specificity of the antigen is examined by testing it against sera from cattle neither infected with pleuro-pneumonia nor recently vaccinated against it but suffering from as large a variety of other diseases as possible.*

(vi) As a final testing of the antigen, the whole of a day's test is repeated using the new antigen, when the results must coincide with those obtained with the known satisfactory antigen.

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* As will be discussed later, care must be taken that animals considered free from pleuro-pneumonia are really so; they should not have been inoculated against the disease more recently than 2 months before collection of blood.

11. The Test Proper.

(i) *The Diagnostic or "Four-tube" Test.*

The contents of each tube during the period of fixation are as follows:—

Tube 1 (nearest the observer).—One volume of a 1 in 10 saline dilution of inactivated suspect serum, one volume of saline containing 2½ M.H.D. complement, and one volume of antigen dilution containing 5-10 A.U.

Tube 2.—One volume of a 1 in 20 dilution of inactivated suspect serum, one volume of saline containing 2½ M.H.D. of complement, and one volume of antigen.

Tube 3.—One volume of a 1 in 30 dilution of inactivated suspect serum, one volume of saline containing 2½ M.H.D. of complement, and one volume of antigen.

Tube 4 (serum control).—One volume of saline, one volume of a 1 in 10 dilution of inactivated suspect serum, and one volume of saline containing 2½ M.H.D. complement.

The order of adding reagents is:—

(1) To tubes 2 and 4, add one volume of saline.

(2) To tube 3, add two volumes of saline.

(3) To tubes 1, 2, 3, and 4, add one volume of the 1 in 10 dilution of inactivated suspect serum and mix.

(4) From tubes 3 and 2, remove and discard two and one volumes of fluid respectively, leaving the serum dilutions described above.

(5) To each of the four tubes, add one volume of complement (2½ M.H.D.).

(6) To tubes 1, 2, and 3, add one volume of antigen dilution containing 5-10 A.U.

The racks are well shaken after each fresh reagent is added, and are finally placed in the water-bath at 37.5°C., shaking well every 10 minutes, and after 30 minutes one volume of sensitized sheep red-cell suspension is added to each tube. Each rack is well shaken immediately after addition of cells and placed on the bench until all racks are completed. A slow worker may find it advantageous to place each rack in the bath as completed, making a note of the time, instead of accumulating them all on the bench. They are then re-shaken and replaced in the water bath, shaken every 10 minutes and removed and read after 30 minutes. The series of tests should commence with two known-negative sera and a known-positive serum as controls.

There should also be:—

(i) A red-cell control tube containing three volumes of saline and one volume of sensitized red-cell suspension, the cells being added during the general addition. In this there should be no haemolysis, thus indicating that no deterioration has occurred since the complement titration, when a red-cell control tube was also employed in the test.

(ii) Antigen-control tube containing one volume of saline, one volume of complement dilution ($2\frac{1}{2}$ M.H.D.), and one volume of antigen, to which is added one volume of sensitized red cells during the general addition. Complete haemolysis must occur. The requirements of all control sera and tubes being fulfilled, the results with the suspect sera may be recorded.

(ii) *Complete Titration of Positive Sera.*

For many purposes, e.g., experimental work, it is necessary to determine the full titre of antibody in the sera. The technique is similar to that already described, but the serum dilutions are carried out to the required limit, which may be 1 in 200, 1 in 500 or even more, the tube series of course running from left to right and not away from the observer as in the diagnostic test. The dilutions, made by the teated-pipette method as usual, run 1 in 10, 1 in 20, 1 in 40, 1 in 60, &c.*. At the end of the series, a serum-control tube, to which a 1 in 10 dilution of serum is added, is included.

12. Method of Recording Results.

++++ = complete fixation of complement, i.e., no haemolysis.

+++ = practically complete fixation of complement, i.e., a slight trace of haemolysis.

++ = partial fixation of complement, i.e., partial haemolysis.

+ = very slight fixation, i.e., practically complete haemolysis.

± = extremely slight fixation, i.e., almost undistinguishable from negative, but with a slight opacity.

— = no fixation, i.e., complete haemolysis.

In order to save space, reactions will be referred to throughout the text in the form of a fractional number of which the numerator, ranging from o to iv, represents the number of positive signs and the denominator the dilution of serum with which the reaction indicated by the nominator was obtained; thus iv/200 means a ++++ reaction in a serum dilution of 1 in 200; i/10 means a + reaction in a dilution of 1 in 10; o/10 means a — reaction in a dilution of 1 in 10. An uncompleted test, in which the serum dilutions employed were not sufficient to determine the full titre of detectable antibody, is indicated by a line drawn over the dilution; thus, iv/$\overline{200}$ means that a ++++ reaction was obtained in a serum dilution of 1 in 200, but that the series of dilutions was not carried out further; the titre would probably have gone considerably higher.

IV. EXPERIMENTAL INVESTIGATIONS.

1. General.

(i) *Specificity and Normal Antibody.*

It is, of course, extremely important on the one hand that animals infected with the pleuro-pneumonia organism should always give positive complement-fixation reactions, and that on the other hand both healthy normal animals and those suffering from all other pathological conditions likely to be encountered in practice should give negative reactions.

* We have subsequently adopted a geometrical series of dilutions, each twice that of the preceding tube, as being more informative and less troublesome (i.e., 1 in 10, 1 in 20, 1 in 40, 1 in 180, 1 in 160, &c.).

During the course of the investigation at Townsville, no non-specific positive reactions have been obtained from the sera of pleuro-pneumonia-free animals suffering from the following varied conditions:—tuberculosis, piroplasmosis, babesiellosis, anaplasmosis, theilerasis (*T. mutans*), lantana poisoning (*L. sanguinea*), echinococcosis, cysticercosis (*C. tenuicollis*), severe intestinal parasitism (*Haemonchus, Ostertagia, Cooperia, Monodontus,* and *Oesophagostomum*), septic pneumonia, pleurisy of undetermined origin (but not pleuro-pneumonia); aphosphorosis, infectious rhinitis, contagious ophthalmia, vaccinia, and various neoplastic diseases. In addition, females in various stages of pregnancy up to full term have been included.

In order to investigate further the specificity of this reaction, it was decided to test at Townsville the sera of animals slaughtered at the Launceston Municipal Abattoirs, Tasmania, in which State, as is known, pleuro-pneumonia does not exist. It may be stated that, out of 2,062 sera tested, 2,059 have given no detectable fixation in a 1 in 10 dilution, while only 3 have given a i/10 reaction, and none a ii/10 or greater. Of these animals, 1,640 were reported by the Veterinary Inspector as showing no abnormalities *post mortem*, and among the remaining 422 the following conditions were noted:—tuberculosis, brucelliasis, forage poisoning (parabotulism), cysticercosis (*C. tenuicollis*), and echinococcosis of the lungs, various neoplastic diseases, traumatic pericarditis, abscess in the liver, abscess in the head and tongue, actinobacillosis, actinomycosis, "plant poisoning," septic peritonitis, septic wounds, chronic mastitis, pleurisy, and nephritis.

Whether any diseases or conditions exist that may produce false-positive reactions to this complement-fixation test, we do not know; if so, they are entirely outside our experience.

From the above, it appears that, in the absence of prophylactic vaccination or infection (see later), the great majority of cattle, whether healthy or suffering from a large variety of other diseases, have no detectable natural antibody in their sera in a dilution of 1 in 10; only 3 out of 2,062 (= 0.145 per cent.) gave even a i/10 reaction, which, as we shall see later, has some diagnostic significance in animals previously negative. There were no ii/10, iii/10, or iv/10 reactions. The test, therefore, appears to be highly specific.

(ii) *Anti-complementary Sera.*

During the weekly testing of the sera of animals under prolonged observation, we have encountered anti-complementary sera on rare occasions. The fixation of complement in the serum-control tube was occasionally complete, but was usually only partial or slight. With one exception, blood serum collected a few days later for a re-test always gave complete haemolysis in the serum-control tube, i.e., the anti-complementary property of the blood serum was usually only temporary.

Three sera out of the 2,065 sera from Tasmania showed slight anti-complementary properties, two giving ++ reactions, the third a +. With anti-complementary sera, the first tube, containing a 1/10 dilution of serum *plus* antigen, may, because of the antigen*, give a stronger fixation than the serum-control tube. A ii/10 reaction in the

* The antigen may itself absorb a small amount of complement (see footnote p. 20).

test with a + serum control should be regarded as negative pending a re-test, but when the reaction is iv/10, iv/20, or iv/30 in the test with + in the serum control, then the animal should be regarded as positive pending a re-test.

Sera with higher anti-complementary properties than + should always be subjected to a re-test before a report is furnished.

2. Complement-fixation Response to Prophylactic (Tail) Vaccination.

Our earlier experiences and Gregory's (1927) had indicated that the artificial infection of cattle in the subcutaneous tissue at the tip of the tail, i.e., the commonly practised vaccination of Willems, gave rise to complement-fixing antibody in the serum at a titre commonly associated with natural pulmonary infection; Hindmarsh (1934) has confirmed this. In other words, a positive complement-fixation reaction in an animal recently vaccinated had no significance for the diagnosis of pleuro-pneumonia. The observations, however, were fragmentary, and nothing was known of the extent of the post-vaccination complement-fixation reaction, nor its persistence.

It was therefore decided to investigate these important and fundamental questions by testing the sera of cattle at regular close intervals from before vaccination until the disappearance of circulating antibody. Accordingly, 33 cattle were inoculated subcutaneously at the tip of the tail with culture vaccine grown in V.F.–O.S. broth* (Turner, Campbell, and Dick, 1935), and 12 with "natural virus," i.e., pleuritic exudate from natural cases of pleuro-pneumonia, the dose in all cases being 0.2 ml. Before inoculation, all animals were completely negative to the complement-fixation test. Blood samples for testing were withdrawn thereafter every two days (three days when week-ends intervened) until the sera became negative again.

The results are set out in Table 1. It will be observed that, whilst there was a certain degree of individual variation of response as regards onset, maximal titre, and disappearance of circulating antibody, there nevertheless, was a general similarity. A typical complement-fixation chart of one of the more persistent reactors is shown in Fig. 1.†

By determining the range of titre in tail-inoculated cattle and the period during which serological response was detectable, it was hoped that information would be obtained which would permit the reaction to be interpreted with confidence even in animals that had been previously submitted to the widely-used Willemsian vaccination.

From Table I., it will be observed that vaccination with culture vaccine led to the appearance of detectable antibody (i.e. i/10) as early as the second day in some cases, although the majority were between 3 and 8 days, and one was delayed until the eleventh day. Frankly positive reactions (i.e., iv/10 or more) were reached generally between the sixth and tenth days, with a range between 4 and 12 days. The iv/10 reaction persisted until between the tenth and twenty-eighth days, and all trace of reaction had disappeared by the thirty-seventh day, the majority terminating about the twenty-fifth day, and one as early as the twelfth day. The maximal titres ranged from iii/10 to iv/200.

* See page 74. † See page 76.

TABLE 1.—CORRELATION BETWEEN VACCINE, TAIL REACTION, AND COMPLEMENT-FIXATION REACTION IN CATTLE VACCINATED AGAINST PLEURO-PNEUMONIA BY THE WILLEMSIAN METHOD.

No.	Day on which antibody first appeared (at least i/10).	Day on which iv/10 reached.	Day on which maximal titre reached.	Maximal titre.	Last day iv/10.	First day o/10.	Tail reaction.
			(a) Culture Vaccine.				
365	6	8	10	iv/30 ; i/70	16	26	A
379	6	6	8	iv/70 ; i/100	26	35	A
383	6	10	10	iv/10 ; i/40	10	24	A
378	4	6	8	iv/70 ; i/100	20	25	A
369	8	10	10	iv/10 ; i/40	13	22	A
356	8	..	10	iii/10 ; i/30	..	17	A
349	8	10	10	iv/10 ; i/40	10	17	A
293	4	6	6	iv/30 ; i/60	27	36	A
362	6	9	9	iv/10 ; i/30	9	13	A
370	7	..	10	iii/10 ; i/30	..	12	A
367	7	12	12	iv/10 ; i/40	12	16	A
384	5	10	10	iv/10 ; i/40	10	14	A
380	4	6	11	iv/60 ; i/90	15	23	A
366	4	8	8	iv/10 ; i/40	15	26	A
252	7	7	7	iv/30 ; i/60	11	14	B
341	2	7	9	iv/60 ; i/90	16	23	B
339	5	5	5	iv/20 ; i/60	10	12	B
344	5	5	5	iv/20 ; i/60	10	14	B
354	5	5	10	iv/50 ; i/90	19	33	B
376	11	11	11	iv/10 ; i/30	11	16	B
372	5	7	9	iv/40 ; i/70	21	30	B
374	5	7	7	iv/80 ; i/100	29	23	B
268	4	6	6	iv/60 ; i/90	28	..	B
377	6	6	10	iv/40 ; i/70	15	27	B
373	6	6	17	iv/30 ; i/70	22	29	B
268	6	6	10	iv/60 ; i/90	20	27	B
288	3	10	15	iv/60 ; i/90	28	32	B
230	3	7	15	iv/70 ; i/100	22	32	B
244	3	7	13	iv/90 ; iii/100	22	32	B
239	3	7	13	iv/80 ; ii/100	26	32	B
337	2	4	9	iv/160 ; ii/200	16	23	C
364	6	6	10	iv/$\overline{200}$	31	37	C
386	6	9	13	iv/$\overline{200}$	30	34	C
			(b) Pleuritic-exudate Vaccine ("Natural virus.")				
15	8	10	10	iv/20 ; i/50	10	29	A
311	8	8	8	iv/40 ; i/70	13	20	A
312	8	8	10	iv/40 ; i/60	20	27	A
316	8	10	8	iv/40 ; i/60	17	28	A
319	6	10	10	iv/10 ; i/40	10	23	A
321	8	10	10	iv/10 ; i/40	10	22	A
30	8	22	22	iv/10 ; i/40	22	36	A
322	8	..	10	iii/10 ; i/30	..	22	A
2	6	8	8	iv/30 ; i/60	15	30	B
12	6	8	10	iv/40 ; i/70	13	20	B
14	6	8	10	iv/40 ; i/70	13	29	B
37	8	8	15	iv/30 ; i/70	24	26	B

NOTES.

Key to Tail Reactions.

A = Maximal swelling extending about 2 inches from the tip of the tail.
B = Maximal swelling extending about 3 inches from the tip of the tail.
C = Maximal swelling extending about 4 inches from the tip of the tail.

There appeared to be a general correlation between the degree of local reaction at the site of inoculation in the tail and the maximal complement-fixation titre, but there were some anomalies; thus, one animal (No. 380), that gave a local reaction very difficult to detect gave a maximal complement-fixation reaction of iv/60 and i/90, whereas one that gave a good tail reaction (No. 376) gave only iv/10 and i/30. Another animal with an A-type tail reaction (No. 370) gave only iii/10 and i/30.

With "natural virus," detectable antibody (i/10) first occurred from the sixth to the eighth day, the first iv/10 reaction from the eighth to the twenty-second day; the maximal titres varied from iii/10 to iv/40, and occurred from the eighth to the twenty-second day. The iv/10 reaction persisted until between the thirteenth and the twenty-fourth day, and the animals became completely negative between the twentieth and the thirty-sixth day.

A surprising fact is the rapidity with which maximal titre is attained after the first appearance of antibody in the blood, the equally rapid fall that sometimes occurs, and the comparatively short period during which antibody is detectable. Occasionally, an animal has given an o/10 reaction on the sixth day and a iv/20, i/50 on the eighth day; one animal (No. 37) gave a iv/20, i/60 reaction on the twenty-fourth day, and two days later was completely negative.

Of great importance, as well as interest, is the fact that the maximal duration of a full-positive reaction (iv/10) was 31 days after inoculation, and that all of the 45 cattle examined were completely negative after 37 days.

It is therefore probable that all complement-fixation reactions due to prophylactic tail-inoculation may be regarded as having passed after about 5½ weeks. As a further safeguard, we recommend a total delay of 8 weeks after prophylatic vaccination before testing. In making this recommendation, it is assumed that tail reactions have been normal in onset and degree of reaction, these being the type of response to which we are accustomed. We have had no experience with the delayed and often serious tail reactions that have been reported by some other workers (Robin, 1925); complement-fixation reaction might possibly persist longer than 8 weeks in such cases.†

Summary.—Cattle prophylactically vaccinated at the tip of the tail with either cultures in V.F.–O.S. broth or pleuritic exudate ("natural virus") subsequently gave iv/10 complement-fixation reactions, and often much stronger, within 4 to 22 days. No traces of antibody were found in the circulating blood after the thirty-seventh day. It is considered that this complement-fixation reaction may be confidently applied to cattle for diagnostic purposes, provided they have not been prophylactically inoculated less than 8 weeks previously, and that the local reaction has not been of the invasive type.

3. Complement-fixation Response to Inoculation by Various Routes.

For experimental purposes, it has been necessary to inoculate cattle by various routes. As most were killed at the height of their reactions, the records are necessarily incomplete. The following observations have been accumulated.

† We have since examined cattle suffering from excessive reactions accompanied by sloughing of considerable portions of the tail, but only when the gluteal muscles were invaded did the reaction persist longer than 6 weeks.

(*a*) *Subcutaneous Inoculation.*—The majority were inoculated in the prescapular region, but a few behind the scapulo-humeral articulation. Generally "natural virus" was used, but occasionally culture. The reactions, with the exception of No. 212, were of the usual type, i.e., large, painful, oedematous swellings up to 20 cm. by 15 cm. Their serological responses are shown in condensed form in Table 2.

TABLE 2.—SEROLOGICAL ANALYSIS OF CATTLE SUBCUTANEOUSLY INOCULATED WITH EITHER PLEURITIC EXUDATE OR CULTURE OF THE ORGANISM (DOSE 5–10 ML.).

No.	Day on which antibody first appeared (at least i/10).	Day on which iv/10 reached.	Day on which maximal titre reached.	Maximal titre.	Last day iv/10.	First day o/10.
50	5	7	23	iv/90, iii/100 ..	n.d.	n.d.
51	7	10	14	iv/80, ii/100 (killed) ..	..	..
54	7	10	16	iv/70, ii/100 ..	n.d.	n.d.
55	3	5	16	iv/100 (killed) ..	..	..
57	7	7	16	iv/200	..	..
58	5	7	14	iv/100	n.d.	n.d.
62	7	10	21	iv/100	n.d.	n.d.
31	2	4	7	iv/40, i/80	24	26
63	2	9	n.d.	n.d.	n.d.	n.d.
215	24	24	37	iv/80, i/160	51	58
318*	31	31	37	iv/200 (killed) ..	..	..
319*	24	24	25	iv/200 (killed) ..	..	..
88	6	6	9	iv/200 (killed) ..	..	..
56	5	7	12	iv/70, i/100 (killed) ..	..	..

n.d. = not determined.

* Both these animals were inoculated with 10 ml. of a V.F.-O.S. culture that had been repeatedly sub-cultured in V.F.-O.S. for two and a half years.

(*b*) *Peritracheal Inoculation.*—During experiments on the attempted artificial reproduction of the pulmonary disease, certain cattle were inoculated by Cameron's method (1906), i.e., deeply into the peritracheal fascia at the entrance of the trachea into the thorax. With the exception of No. 52 (inoculated with pleuritic exudate), all were inoculated with culture. There was generally a very marked local oedematous reaction, and, *post mortem,* some of the animals showed large quantities of exudate in the pleural cavities, but no characteristic pneumonic lesions. Their serological responses are shown in Table 3.

TABLE 3.—SEROLOGICAL ANALYSIS OF CATTLE INOCULATED IN THE LOWER CERVICAL PERITRACHEAL FASCIA.

No.	Day on which antibody first appeared (at least i/10).	Day on which iv/10 reached.	Day on which maximal titre reached.	Maximal titre.	Last day iv/10.	First day o/10.
224	11	60	60	iv/40, i/100 ..	Still iv/20 when killed on 120th day	..
231	11	18	18	iv/100, i/160 (killed)	..	..
393	7	14	14–18	iv/200 (killed) ..	..	..
52	7	10	19	iv/100 (killed) ..	..	..

No. 224 remained a persistent reactor for two months, after which it was killed, and showed no lesions *post mortem*. The organism was not recovered from any organ examined. It is probable that an undetermined focus of infection existed.

(*c*) *Intrapulmonary Inoculation.*—Three cattle were inoculated into the lungs with "virus" either mixed with calcium chloride or followed by artificial pneumo-thorax (Nos. 3, 97, 96); another animal (345) received a coarse suspension of an acute lung-lesion. Their serological responses are set out in Table 4.

TABLE 4.—SEROLOGICAL ANALYSIS OF CATTLE INOCULATED INTRAPULMONARILY.

No.	Day on which antibody first appeared (at least i/10).	Day on which iv/10 reached.	Day on which maximal titre reached.	Maximal titre.	Last day iv/10.	First day o/10.
3	7	7	9	iv/$\overline{200}$ ..	145	Killed on 250th day when iii/10, i/30
97	2	7	15	iv/40, i/70	41	105
96	4	9	9	iv/40, i/70	104	128
345	6	6	20	iv/$\overline{200}$ ..	Killed on 35th day when iv/80, i/140	..

(*d*) *Intratracheal Inoculation.*—With the exception of numbers 214 and 219, which received culture, the animals described in Table 5 were inoculated with pleuritic exudate by means of a catheter passed down the trachea.

TABLE 5.—SEROLOGICAL ANALYSIS OF CATTLE INOCULATED INTRATRACHEALLY.

No.	Day on which antibody first appeared (at least i/10).	Day on which iv/10 reached.	Day on which maximal titre reached.	Maximal titre.	Last day iv/10.	First day o/10.
69	5	39	39	iv/20, iii/30 ..	39	47
71	7	9	12	iv/$\overline{200}$	56	90
79	7	7	12	iv/$\overline{200}$	Killed on 19th day; iv/80, iii/$\overline{200}$	..
82	9	16	34	iv/$\overline{200}$	98	121
84	8	8	15	iv/$\overline{200}$	Killed on 212th day, when iv/20, ii/30	..
90	12	12	14	iv/80, i/160 ..	48	68
95	7	7	11	iv/$\overline{200}$	Killed on 18th day, when still iv/$\overline{200}$	..
214	5	5	17	iv/$\overline{200}$	77	85
220	40	40	47	iv/$\overline{200}$	82	89
219	12	12	15	iv/$\overline{200}$ (killed) ..	..	..
218	19	19	26	iv/180, iii/200 (killed)	..	..

(*e*) *Intravenous Inoculation.*—For experimental purposes, a small number of cattle have been inoculated into the jugular vein with various specifically infected materials, as set out in Table 6. Their blood sera were tested weekly.

TABLE 6.—SEROLOGICAL ANALYSIS OF CATTLE INOCULATED INTRAVENOUSLY.

No.	Inoculum.	Day on which antibody first appeared (at least i/10).	Day on which iv/10 reached.	Day on which maximal titre reached.	Maximal titre.	Last day iv/10.	First day o/10.
342	Blood culture *plus* agar	7	14	21	iv/80, i/140	Killed 31st day, when iv/10, i/40	..
271	Blood culture *plus* agar	14	14	14	iv/80, i/140	21	Killed 35th day, when i/10
298*	Blood culture alone	7	28	28	iv/20, i/60 (killed)	..	..
353	Blood culture alone	..	..	..	..	..	..
340	Emulsion infected lung	7	7	14	iv/40, i/100 (killed)	..	..
351	Emulsion infected lung	7	7	14	iv/140, i/200	Killed 21st day, when iv/20, i/180	..
274	Emulsion infected lung *plus* agar	14	21	21	iv/160, ii/200	Killed 35th day, when iv/100	..

* Developed a local infection in the subcutis at site of inoculation.

(*f*) *Miscellaneous.*—One animal was inoculated intraperitoneally, another intratesticularly. Their reactions are shown in Table 7.

TABLE 7.—MISCELLANEOUS.

No.	Inoculum.	Day on which antibody first appeared (at least i/10).	Day on which iv/10 reached.	Day on which maximal titre reached.	Maximal titre.	Last day iv/10.	First day o/10.
61	Subcutaneous exudate intraperitoneally	6	11	20	iv/$\overline{100}$ ..	Discharged 36th day, when iv/60, i/100; was o/10 on 66th day	..
70	Pleuritic exudate intratesticularly	7	..	14	iii/10, i/40	..	21

(*g*) In addition, a number of sheep and goats have been inoculated subcutaneously. They all reacted locally, the sheep more strongly than the goats. In all cases, high-titre complement-fixation reactions were obtained. Details are to be published elsewhere in due course.

Summary.—Cattle inoculated by a large variety of routes gave complement-fixation reactions, frequently of a very high titre. Sheep and goats similarly gave positive complement-fixation reactions following subcutaneous inoculation. The reaction is therefore a very useful adjunct to experimental work on pleuro-pneumonia.

4. Complement-fixation Response to the Naturally-Acquired Pulmonary Infection.

(i) *Laboratory Herd.*

The investigation of the sera of cattle artificially inoculated by various routes with the causal organism of pleuro-pneumonia had enabled us to establish provisionally certain criteria of what constitutes a "positive" reaction in those types of infection, but, as is known, it is usually considered impossible to reproduce, by artificial means, the typical pulmonary disease that occurs under natural conditions*; to reproduce pleuro-pneumonia it is necessary to place susceptible cattle in contact with those suffering from the disease. Accordingly, it was decided to establish an infected herd of cattle at the laboratory and to carry out regular testing of their sera. In this way it was hoped to follow the rise and fall of complement-fixing antibody over the whole period of infection and recovery.

The routine testing, clinical observation, and subsequent post-mortem examination were expected to yield information that would enable us to interpret the complement-fixation reaction as a means of diagnosing both acute and chronic pleuro-pneumonia.

(*a*) *Method.*—Opportunity to commence the experiment occurred on the 1st November, 1932, when the disease broke out in a dairy herd 2 miles from the laboratory. The diagnosis by Dr. J. Legg of this Station was based on the post-mortem appearances of two dead cows, and the next day the whole herd of 83, including bulls and calves, were tested and 18 reactors found.

Three acute clinical cases that had given strong positive reactions were removed to the laboratory and placed in contact with 32 uninfected animals, comprising 12 "clean" animals (i.e. animals that had never had the disease, been inoculated against it, nor exposed to it), 6 animals vaccinated by tail-inoculation about 4 weeks previously, and 14 vaccinated by various other methods. The herd was confined in a small isolated buffered paddock (about 5 acres in area), being placed in very close contact in a smaller portion of the paddock (about 1.2 acres) for eight hours a day. Contact was further encouraged by passing the animals through a crush for the taking of rectal temperatures twice daily. These conditions continued for two months, at the end of which the animals were allowed to graze over an area of 40 acres in the day-time, and brought back into the small compound at night.

The first case of pleuro-pneumonia occurred 48 days after exposure to infection, in an animal (No. 31) that had been vaccinated by subcutaneous inoculation 52 days before exposure; it had given marked local and moderate complement-fixation reactions but recovered and had been quite negative for three and a half weeks before introduction into the infected environment (Figs. 18 and 23). Thereafter, further cases occurred at intervals, and, from time to time, unvaccinated or vaccinated calves were introduced into the herd to replenish the numbers. The age of the experimental animals was mostly from 10 to 15 months when introduced into the experiment.

The disease having apparently subsided about 8½ months after the beginning of the experiment, an opportunity of adding a fresh source

* We have recently succeeded in reproducing the typical pneumonic infection by exposing cattle to finely atomized culture. Details will be published later.

of contagion was availed of in late September, 1933, i.e. about $10\frac{1}{2}$ months after the beginning of the experiment, when an outbreak occurred in another neighbouring dairy herd. Five acute cases were placed with the animals in the buffered paddock, with another addition of 15 "clean" animals. About $7\frac{1}{2}$ weeks later, the disease broke out again, and cases occurred until the 11th April, 1934. Since that date, the experiment has been gradually concluded by the killing and examination of the survivors.

Altogether, 8 clinical cases of pleuro-pneumonia and 89 uninfected animals were placed into prolonged contact during the experiment. The uninfected animals were added as follows:—

2/11/32	:	32, comprising 12 "clean", 6 tail-vaccinated, and 14 variously vaccinated.
19/11/32	:	10 "clean".
1/1/33	:	4 "clean".
26/4/33	:	6 "clean".
26/6/33	:	7 "clean".
3/8/33	:	3 tail-vaccinated.
16/8/33	:	12 "clean".
22/9/33	:	15 "clean".

From the beginning of the experiment, all the animals were placed on a routine weekly complement-fixation test, the serum dilutions being 1 in 10, 20, and 30. Reactors were then usually tested to higher dilutions. Blood was taken by means of wide-bore needles from the jugular vein, and subsequently treated as described in section III.

Furthermore, the rectal temperatures were taken twice daily until December, 1933, after which they were taken once daily (mornings only), and reactors were placed under close clinical examination.

Besides the above 97 animals, 8 more cases from neighbouring outbreaks diagnosed by means of the complement-fixation test were brought to the laboratory, including cattle at a very early pre-clinical stage, acute clinical cases, resolving cases, and one that gave the type of reaction we had learned to associate with chronic "carriers".

(*b*) *Results.*—All the cattle referred to, a total of 105, were eventually examined *post mortem*. The result was that 65 out of 82 previously "clean" animals and 5 out of 23 vaccinated animals (i.e. 70 out of the 105) showed signs of active, chronic, or healed pleuro-pneumonia*, depending upon the stage of the disease at which the autopsies happened to be performed. Most had been diagnosed clinically as cases of pleuro-pneumonia, some had been further confirmed by blood culture or lung puncture, but some had been extremely mild cases (Figs. 19, 20, and 23).

Sometimes, the post-mortem examinations were purposely delayed for many weeks, or even months, after the animals had passed their acute stage, and consequently healing had often progressed very far.

* As far as the infection experiment at the laboratory was concerned, i.e., excluding cases brought to the laboratory from neighbouring farms, 18 out of 23 (78.26 per cent.) vaccinated cattle were resistant to infection and only 17 out of 65 unvaccinated (26.14 per cent.). The standard deviation of the difference (52.12 per cent.) is 11.87 per cent., the ratio of the difference to its S.D. is 4.39, and hence the difference is of an extremely high order of significance.

Especially when the disease had been of a very mild nature, frequently the only signs of earlier acute pleuro-pneumonia were small patches of thickened visceral or parietal pleura (*pleuritis fibrosa*) with a few delicate fibrous adhesions between lungs and thoracic wall, diaphragm, or pericardium, indicative of an earlier acute exudative pleuritis; sometimes, in addition, there was slight fibrous thickening of interlobular septa in certain regions of the lungs, indicating an earlier, interlobular, sero-fibrinous lymphangitis with resulting fibrosis. It was soon realized that one needed considerable experience in the post-mortem examination of animals recovered from pleuro-pneumonia in order to appreciate the high degree of recovery and resolution that can take place, and that, if apparent discrepancies between the results of a positive test and post-mortem examination were to be avoided, the interval between them must be as short as possible.

In addition to the acute, resolving, and healed cases, 9 chronic cases, i.e., "carriers," were found in this experimental herd, at post-mortem examination (Nos. 22, 31, 89, 166, 168, 193, 282, 303, 308). Three other cattle (Nos. 23, 35, 270) that had given positive complement-fixation reactions at the time of their illness but negative reactions when killed, had small sequestra about 1 cm. in diameter in the lungs; but cultural tests and inoculation into susceptible calves (in the case of Nos. 23 and 35) showed them to be sterile. As the ability of the test to detect chronic carriers is of great practical importance, the above cases are grouped together for ease of study in Table 8.

It was found that complement-fixing antibody appears in the blood stream of naturally infected animals often before the onset of clinical symptoms and, as was the case with artificially inoculated animals, may reach a high titre* very rapidly (Figs. 2, 3, 5, 7, 8, 10, 13, 14, 16, and 18). An animal giving a iv/10, iii/20, ii/30 reaction often gave complete fixation in a dilution of 1 in 200 (i.e. iv/200) two days later. In severe but non-fatal cases, the iv/200 reaction persisted from four to eight weeks (Figs. 13, 15, and 18).

After the cessation of the clinical symptoms, two types of complement-fixation charts were obtained.

The first type showed a gradual diminution of circulating antibody, i.e., the titre gradually dropped until a fairly persistent reaction was obtained usually of the "iv/10," "iv/20," or "iv/30" type but occasionally stronger (up to iv/100) or weaker (iii/10, ii/20, i/30). These reactions usually persisted until the animals were finally destroyed many months later, when they were found to have one or more encapsulated specifically infected sequestra in their lungs (Figs. 4, 12, 13, 14).

The second type of chart showed a more rapid diminution of antibody. The reaction soon dropped to a iii/10, ii/20, i/30, and then, after a few weeks, became negative with perhaps an intermittent ii/10 or i/10 reaction (Figs. 5, 6, 7, and 8). The post-mortem findings in animals giving this second type of chart were entirely different from the former. Pleural adhesions and possibly fibrosis of a portion of one of the lobes

* Several iv/440 and even iv/640 reactions have been obtained from sera collected immediately before death.

TABLE 8.—PARTICULARS OF CARRIERS (CASES WITH INFECTED PULMONARY SEQUESTRA) AND PSEUDO-CARRIERS (CASES WITH STERILE SEQUESTRA) AMONG THE EXPERIMENTAL HERD.

No.	Complement-fixation Reaction at Death.	Nature of Lesions.	Cultures.	Remarks.
22	iv/20, iii/30	Two thin-walled sequestra 5 cm. and 6 cm. in diameter, contents fluid	+	
31	iii/10, ii/20, i/30	Sequestrum 10 cm. x 8 cm.	+	Complement-fixation reaction the previous week was iv/10, iii/20, ii/30
89	iv/40, iii/60, ii/80, i/100	Two large well-encapsulated sequestra almost comprising whole right lung	+	
166	iv/40, iii/60, ii/80, i/100	Sequestrum 5 cm. x 5 cm.; well encapsulated	+	
168	iv/100, iii/120, ii/140, i/180	Sequestrum 10 cm. x 8 cm.; well encapsulated	+	
193	iv/10, ii/20, i/30	Sequestrum 1 cm. x 1 cm.	+	
282	iv/60, iii/80, ii/100	Two sequestra, one "large" and one "small"	+	
303	iv/30, iii/40, ii/60, i/80	Large sequestrum comprising whole left diaphragmatic lobe	+	
308	o/10	Encapsulated sequestrum 4 cm. x 3 cm.	+	Strong positive reactor for nine months; then inoculated intradermally with culture; thereafter declined to negative; killed two months after inoculation (see Fig. 17)
23	o/10 ..	Small sequestrum 0·5 cm. x 1 cm.	—	Animal inoculation negative
35	o/10 ..	Small sequestrum 1·5 cm. x 1 cm.	—	Animal inoculation negative
270	o/10 ..	Small sequestrum 0·5 cm. in diameter	—	Animal inoculation not performed

of the lungs were all the pathological changes found. The lung tissue, mediastinal and bronchial lymph glands, and other organs of such animals were sterile.

(*c*) *Transient reactions.*—The routine weekly testing led to the recognition of a curious phenomenon that would otherwise probably have been missed, namely, the occurrence of one or more transient complement-fixation reactions of a weak or moderately strong titre in certain animals both in the resistant and susceptible groups.

For the purpose of this discussion, the experimental herd may be regarded as consisting of six groups, viz.:—

1. Unvaccinated susceptible (40), i.e., subsequently developed pleuro-pneumonia.
2. Unvaccinated resistant (14), i.e., did not develop it.
3. Vaccinated susceptible (5).
4. Vaccinated resistant (20).†
5. "Intradermal" susceptible* (9).
6. "Intradermal" resistant* (2).

Transient reactions occurred in all groups except 6, which, however, contained only two animals. They were of four types:—(i) weak (i.e., ii/10 or iii/10); (ii) intermittent weak, in which this weak type was repeated once or twice; (iii) strong (i.e., iv/10 or greater); and (iv) intermittent strong, in which the reactions were repeated once or twice, one at least being iv/10 or greater. It will be noticed that the reactions were of sufficient titre (ii/10) or greater) to warrant a positive diagnosis, and therefore might be a source of apparently false positives; their significance will be discussed later.

An analysis of the transient reactions, showing their distribution in the above groups, is given in Table 9, and the salient particulars of the animals concerned in Table 10.

TABLE 9.—ANALYSIS OF TRANSIENT REACTIONS.

Group.	No. of Animals.	Number with Transient Reactions.				Total.	Total with at least one iv/10.
		Weak.	Intermittent Weak.	Strong.	Intermittent Strong.		
1	40	4	0	2	2	8	4
2	14	3	2	0	0	5	0
3A (Tail) ..	2	1	0	0	0	1	0
3B (Subcut.) ..	1	1	0	0	0	1	0
3C (Intratest.) ..	1	0	0	1	0	1	1
3D (Intraperitoneal)	1	0	0	1	0	1	1
4A (Tail) ..	7	3	0	0	1	4	1
4B (Subcut. and peritrach.) ..	6	0	2	0	2	4	2
4C (Intratrach.) ..	4	0	1	0	3	4	3
4D (Intrapulm.) ..	3	0	0	0	1	1	1
5	9	1	0	2	0	3	2
6	2	0	0	0	0	0	0

* These are animals that had been subjected to allergic intradermal tests at various periods before the "transient rises" occurred; they might possibly be included in the vaccinated groups.

† Nos. 69 and 82, which were inoculated intratracheally and became persistent reactors, are not included in this section; they are cases of experimental infection.

TABLE 10.—PARTICULARS OF CATTLE THAT GAVE TRANSIENT REACTIONS.

No.	Date of introduction into infected environment.	Date of transient reactions.	Duration of reaction.	Titre of transient reaction.	Type.	Date of clinical manifestation of C.P.P.
		Group 1 (Unvaccinated susceptible).				
11	19.11.32	8.2.33	Less than one week	iv/10, iii/20, ii/30	Strong ..	14.3.33
89	2.11.32	14.3.33	One week ..	iv/20, ii/60, i/100	Strong ..	2.5.33
85	2.11.32	8.2.33	One week ..	iv/30 ..	Intermittent strong	15.5.33
		9.3.33	Four weeks..	iv/40, ii/60, i/80		
264	16.8.33	26.9.33	Two weeks	iv/10, iv/20, iii/30	Intermittent strong	5.2.34
		30.10.33	<One week ..	ii/10, i/20, i/30		
		11.12.33	<One week ..	iv/10, iii/20, ii/30		
91	2.11.32	2.1.33	<One week ..	iii/10, ii/20, i/30	Weak ..	1.2.33
72	2.11.32	24.1.33	<One week ..	ii/10, i/20	Weak ..	7.3.33
265	16.8.33	30.10.33	<One week ..	ii/10, i/20	Weak ..	22.1.34
292	26.9.33	12.2.33	Two weeks	ii/10, ii/20, i/30	Weak ..	9.4.34
		Group 2 (Unvaccinated resistant).				
75	2.11.32	17.1.33	<One week ..	ii/10, i/20	Intermittent weak	No
		15.3.33	<One week ..	ii/10, i/20		
78	2.11.32	21.11.32	<One week ..	ii/10, i/20	Intermittent weak	No
		20.3.33	<One week ..	ii/10 ..		
189	26.4.33	29.5.33	Two weeks	ii/10, ii/20	Weak ..	No
198	26.4.33	6.6.33	<One week ..	iii/10, ii/20, i/30	Weak ..	No
206	26.4.33	12.6.33	<One week ..	iii/10, ii/20, i/30	Weak ..	No
		Group 3 (Vaccinated susceptible).				
(*a*) Tail— 244	3.8.33	4.9.33	<One week ..	ii/10, i/20	Weak ..	13.11.33
(*b*) Subcutaneous— 31	2.11.32	21.11.32	<One week ..	ii/10, i/20, i/30	Weak ..	21.12.32
(*c*) Intratesticular— 70	2.11.32	14.3.33	Two weeks	iv/10, iii/20, ii/30	Strong ..	4.4.33
(*d*) Intraperitoneal— 61	2.11.32	21.12.32	Three weeks	iv/10, ii/20, i/30	Strong ..	10.7.33
		Group 4 (Vaccinated resistant).				
(*a*) Tail— 14	2.11.32	24.1.33	<One week ..	ii/10, ii/20, i/30	Weak ..	No
15	2.11.32	4.4.33	<One week ..	iv/10, ii/20, i/30	Intermittent strong	No
		8.5.33	<One week ..	iii/10, ii/20, i/30		
		6.6.33	<One week ..	iii/10, ii/20, i/30		
30	2.11.32	7.4.33	<One week ..	iii/10, ii/20, i/30	Weak ..	No
239	3.8.33	11.12.33	<One week ..	iii/10, ii/20, i/30	Weak ..	No
(*b*) Subcutaneous and peritracheal— 43	2.11.32	21.11.32	<One week ..	ii/10, i/20	Intermittent strong	No
		28.3.33	<One week ..	iv/10, ii/20, i/30		
45	2.11.32	21.11.32	<One week ..	ii/10, i/20		
		28.12.32	<One week ..	ii/10, i/20	Intermittent	
		14.3.33	<One week ..	ii/10, i/20		
		11.4.33	<One week ..	iii/10, ii/20		
		18.4.33	<One week ..	ii/10, i/20	Weak ..	No
		8.5.33	<One week ..	ii/10		
		6.6.33	<One week ..	ii/10, i/20		
50	2.11.32	21.11.32	<One week ..	iii/10, ii/20, i/30		
		6.12.32	<One week ..	ii/10 ..	Intermittent weak	No
		2.1.33	<One week ..	ii/10 ..		
		6.6.33	<One week ..	ii/10, i/20		
65	2.11.32	14.3.33	Three weeks	iv/10, iv/20, iii/30	Intermittent strong	No
		18.4.33	..	iii/10, ii/20, i/30		
		4.5.33	Two weeks	iii/10, ii/20, i/30		
		6.6.33	<One week ..	iv/10, iii/20, i/30		

TABLE 10—*continued.*

No.	Date of introduction into infected environment.	Date of transient reactions.	Duration of reaction.	Titre of transient reaction.	Type.	Date of clinical manifestation of C.P.P.
			Group 4—continued.			
(c) Intrapulmonary— 3	6.12.32	21.2.33	Two weeks	iv/10, iv/20, iii/30	Intermittent strong	No
		5.4.33	<One week ..	ii/10, i/30		
		2.5.33	<One week ..	ii/10, i/20		
		6.6.33	<One week ..	iii/10, i/20		
			Group 5 (*"Intradermal" susceptible*).			
227	26.6.33	11.12.33	<One week ..	ii/10, i/30	Weak ..	29.1.34
233	16.8.33	4.9.33	Five weeks*	iv/20, iii/30	Strong ..	11.12.33
234	26.6.33	26.9.33	Two weeks	iv/10, iii/20, ii/30	Strong ..	13.3.34

* This animal became strongly positive (iv/30) on 11.12.33 and remained positive until 30.4.34. Its clinical symptoms then were considered mild. It is possible that the particularly long "transient reaction" (five weeks), although not of a very high titre (iv/20), may have been due to an extremely mild attack of pleuro-pneumonia that escaped clinical detection.

Among 8 out of 40 unvaccinated cattle that subsequently developed pleuro-pneumonia (i.e., group 1), there were 4 weak, 2 strong, and 2 intermittent-strong reactions, from 41 to 139 days after introduction into the infected herd and from 34 to 168 days before the disease ultimately declared itself. In the case of one animal (No. 264) that underwent three transient reactions, the disease became apparent 42 days after the last (Fig. 10). In four of these cases (Nos. 11, 85, 89, and 264) it was of the order (i.e., iv/10 or greater) that we have learned to associate with a frankly positive diagnosis, but whether the causal organism was actually present in these animals at the time of their reactions we do not know, for, unfortunately, no opportunities for determining this have yet occurred. Some evidence bearing on the subject is discussed under "Occult cases."*

"Transient reactions" may indicate the ineffectual efforts of the causal organism to take root in the host, i.e., they may owe their origin to abortive infections, but, if so, the hypothetical mild infections (compare inapparent infections in virus diseases) must, in the case of cattle that nevertheless develop the disease later, frequently be of a non-immunizing or even sensitizing nature. On the other hand, this cannot always be so, for out of 14 unvaccinated animals that did not develop pleuro-pneumonia (group 2), 5 had given transient "weak" complement-fixation reactions (i.e., iii/10 or ii/10), from 35 to 69 days after introduction into the infected environment, and, of these, two gave a second transient action 64 and 119 days later respectively. It should be noted that none of the "unvaccinated resistant" animals gave a transient reaction of iv/10, i.e., a frankly diagnostic reaction, although some approximated to it. If the transient reactions in naturally resistant and susceptible animals are partly due to abortive infections that are able sometimes to immunize them against the "natural" disease, the resistance induced is not always proof against artificial inoculation; for, on being injected subcutaneously with 10 ml. of a culture, two such cattle (Nos. 75 and 206) reacted very severely; No. 206 died, but No. 75 eventually recovered.

* Some further support for the suggestion that the organism may have been present in lung tissue and associated glands at the time of these reactions is given by the work of Yamagiwa, Ito, and Inoue (1932), who recovered it from the apparently healthy lung tissue of an animal giving suspicious complement-fixation reactions, and in addition from the bronchial glands of another.

It is very significant that transient reactions were most frequent when many active cases of pleuro-pneumonia were occurring in the herd. It is possible, therefore, that under these conditions certain animals responded serologically to inhalation of large amounts of the infective agent, without necessarily contracting the *clinical* disease (at that time) or even becoming infected in the strict sense, just as, for instance, they might respond to the absorption of finely pulverized albumen introduced to the pulmonary epithelium by inhalation. According to this view, the inhaled casual organisms need merely be ingested by the pulmonary phagocytes and need not proliferate and invade the body; but the phenomenon was irregular and did not occur in 32 out of 40 unvaccinated animals that subsequently did develop pleuro-pneumonia, nor in 9 out of 14 naturally resistant animals. Further support for this view is the fact that 4 out of 5 vaccinated animals that nevertheless developed the disease (group 3) after introduction into the infected herd gave transient reactions (2 of them being iv/10 reactions) as did 13 out of 20 vaccinated resistant cattle (group 4), these being generally of a much stronger nature, e.g., 7 gave at least one iv/10 transient reaction. Certain of them, especially Nos. 3, 15, 45, 50, 65, and 264, appeared to be extremely sensitive and apt to give repeated reactions of considerable strength. One is reminded here of the phenomenon of secondary response so well known in immunity, as a result of which a small injection of antigen into a previously immunized animal calls forth a rapid and disproportionately large rise in the titre of the circulating antibody. The same phenomenon may have been operating in group 5, in which 3 out of the 9 animals (Nos. 227, 233, and 234) that had previously been inoculated intradermally with killed culture gave transient reactions (two strong and one weak) at intervals of 92, 19, and 48 days, respectively, after introduction into the infected herd; in these cases the intradermal injection possibly sensitized the animals.

(*d*) *Correlation of post-mortem findings with complement-fixation reaction.*—Each of the 70 experimental-herd animals that gave positive post-mortem findings had given a complement-fixation reaction of at least iv/10 and frequently iv/200 or even iv/440 at some period after exposure to infection, associated in most cases with clinical signs (rise of body temperature, &c.). Some of the very mild cases, in which not even a definite thermal response was given, would certainly not have been detected without the aid of the complement-fixation test.

The correlation of the post-mortem diagnosis with the complement-fixation reaction during the sojourn in the infected herd and at the time of death is indicated in the Appendix. It is remarkably good, with only two apparent discrepancies, Nos. 72 and 308 (see Fig. 17). The latter animal has been discussed in Table 8; its post-mortem findings agree well with its earlier reaction (e.g., iv/200 on 3rd October, 1933), and the failure of its serum to fix complement during the last few weeks of its life dated from its intradermal inoculation with a concentrated extract of killed culture, which possibly depleted the blood of circulating antibody.

No. 72, a heifer 12 months old, was exposed to infection on 2nd November, 1932, and gave a complement-fixation reaction of iv/30 on 7th March, 1933. Two days later, it was iv/80 and i/160; thereafter, the reaction declined, the last iv/10 being given on 21st March, 1933.

Blood cultures on the 9th, 10th, 11th, and 13th March, 1933, were negative, but there was a slight febrile reaction near the date of its first iv/10 rise (104° F. on 1st March, 1933, and 105° F. on 4th March, 1934). It was killed on 26th July, 1933, i.e., four months later, but no lesions of pleuro-pneumonia were detected. It is only fair to state, however, that at that time we had not realized how completely pleuro-pneumonia may resolve and how few may be the signs of an earlier acute infection, especially after a lapse of four months. It is possible that a closer search might have revealed scars and very slight adhesions: but, on the other hand, the disease may have been of such a mild nature that complete *restitutio ad integrum* occurred.

(ii) *Cattle Examined at Abattoirs.*

(*a*) *Chronic "carriers".*—Additional comparison of post-mortem findings and the degree of complement-fixing properties of sera from "carrier" cases (i.e., those containing infected pulmonary sequestra) was obtained during the slaughtering operations of the local meat works. The sera from the heart bloods of 31 such animals were obtained, and submitted to the test. Strong positive reactions were given by 30 of the sera, but a negative reaction was obtained from one serum accompanied by the inspector's report, "small encapsulated lesion".

Four out of five samples of sera forwarded to us from Western Australia, from cattle in which chronic lesions were found, gave positive reactions. The negative sample was from an animal reported to be affected with a "large, old encapsulated lesion".

Unfortunately, no cultural examinations were made of the lesions in the lungs of the animals from which all the above sera were obtained, and consequently we are unable to state whether viable organisms were present in the lesions of the two animals that gave negative reactions.

The strength of complement-fixation reaction and the post-mortem reports furnished by the Veterinary Inspectors are set out in Table 11.

(*b*) *Occult Cases.—Specific mediastinal lympho-glandulitis.*—Three peculiar cases were discovered at the abattoirs during the slaughter of a herd of bullocks in which active cases of pleuro-pneumonia were occurring. The only detectable lesion in two of them was a sero-oedematous enlargement of the mediastinal lymph glands, which reached a size of 5 cm. by 11 cm.; on being cut, they resembled the enlarged mediastinal lymphatic glands found in natural cases of pleuro-pneumonia. The other showed, in addition, several small necrotic or semi-caseous areas scattered throughout the oedematous gland. In all cases the lungs appeared absolutely normal.

Blood collected from the heart after slaughter gave strong positive complement-fixation reactions (four-tube "diagnostic" test), i.e., in one case iv/20, ii/30, and in the other two, iv/30.

The enlarged glands were examined at the laboratory, and the first two revealed pure infections with the pleuro-pneumonia organism; the third contained *Mycobacterium tuberculosis* in addition.

These three cases illustrate the sensitivity of this complement-fixation test, and emphasize once more the necessity for very careful post-mortem examination and cultural confirmation of animals diagnosed, by its means, as infected. They also may offer an explanation of certain cases of "transient reactions."

TABLE 11.—COMPLEMENT-FIXATION RESULTS OBTAINED ON SERA FROM "CARRIERS" SLAUGHTERED AT ABATTOIRS.

No.	Serum Dilutions. 1/10.	1/20.	1/30.	Control Serum.	Veterinary Officer's Report.
1	++++	++++	+++	—	"Old pleuro-pneumonic lesion in right diaphragmatic lobe"
2	++++	++++	++++	—	"Encapsulated lesion in left diaphragmatic lobe"
3	++++	++++	++++	—	"Recent encapsulated lesion"
4	++++	++++	++++	—	"Large sequestrum in left diaphragmatic lobe"
5	++++	++++	++++	—	"Sequestrum in left diaphragmatic lobe"
6	++++	+++	++	—	"Encapsulated lesion in right diaphragmatic lobe"
7	++++	++++	+++	—	"Old encapsulated lesion. Mediastinal lymph glands enlarged"
8	++++	++++	++++	—	"Pleuro-pneumonia carrier"
9	++++	+++	++	—	"Old encapsulated lesion"
10	++++	+++	++	—	"Extensive lesion well encapsulated"
11	++++	+++	++	—	"Small encapsulated lesion"
12	++++	++++	+++	—	"Pleuro-pneumonia carrier"
13	++++	+++	++	—	"Pleuro-pneumonia carrier"
14	++++	++++	++++	—	"Encapsulated sequestrum in right diaphragmatic lobe"
15	++++	+++	++	—	"Encapsulated lesion in right diaphragmatic lobe"
16	++++	++++	++++	—	"Large lesion becoming encapsulated in right diaphragmatic lobe"
17	++++	++++	++++	—	"Encapsulated lesion"
18	++++	++++	+++	—	"Old lesion in left diaphragmatic lobe"
19	++++	++++	++++	—	"Large sequestrum"
20	++++	+++	++	—	"Small sequestrum with old adhesions"
21	++++	+++	++	—	"Pleuro-pneumonia carrier"
22	++++	++++	+++	—	"Large sequestrum. Old carrier"
23	++++	+++	++	—	"Small encapsulated lesion"
24	++++	++++	++++	—	"Carrier with large sequestrum"
25	++++	++++	++++	—	"Large lesion becoming encapsulated"
26	++++	+++	++	—	"Small lesions with extensive pleural adhesions"
27	++++	+++	++	—	"Small encapsulated lesion"
28	++++	+++	++	—	"Pleuro-pneumonia carrier"
29	++++	++++	+++	—	"Large sequestrated lesion"
30	++++	++++	+++	—	"Large lesion, encapsulated"
31	—	—	—	—	"Small old encapsulated lesion"

Sera of Chronic Cases received from Western Australia.

No.	1/10.	1/20.	1/30.	Control Serum.	Veterinary Officer's Report.
1	++++	++++	++++	—	"Large encapsulated lesion right lung. Lesion filled with semi-fluid material"
2	++++	+++	++	—	"Encapsulated lesion 13 cms. in diameter in left lung
3	++++	++++	++++	—	"Encapsulated lesion 6 cms. in diameter in right lung"
4	++++	++++	++++	—	"Half right lung affected, showing commencing break-down and encapsulation"
5	+	—	—	—	"Large old well-encapsulated lesion"

(iii) *The Interpretation of the Complement-fixation Reaction.*

As a result of the above work, we are able to lay down the following criteria for interpreting the four-tube test.

—	Dilution.			Interpretation.
	1/10.	1/20.	1/30.	
1	++++	++++	++++	Very strong positive
2	++++	++++	+++	Strong positive
3	++++	+++	++	Positive
4	++++	++	+	Positive
5	+++	++	+	Weak positive
6	++	+	—	Very weak positive
7	+	—	—	Negative
8	—	—	—	Negative

The very strong positive reaction is obtained almost invariably from acute cases, although, occasionally, cattle with large (especially recent) sequestra may give it. Reactions 2, 3, 4, and 5 are obtained either with early cases of pleuro-pneumonia or with chronic cases in which small or old sequestra are present. Reactions 6 or 7 may be given by very early cases, by animals in which sequestra have almost healed, or by almost completely recovered cases in which only adhesions are found, and from which the causal organism has probably disappeared but recently.

The action with regard to the sixth and seventh* types of reactions must depend upon circumstances. If only a single test can be carried out, and the utmost safety is desired, the animals giving such reactions must be dealt with as positives; but this is a contingency that should be accepted only when unavoidable. Every effort should be made to arrange for a re-test, a week later, of the blood serum of animals giving suspicious reactions. If the cattle concerned were merely going through "transient reactions", they might possibly have returned to negative; if they were of the very early type, they would almost certainly have progressed to a much higher titre, for our routine weekly testing brought out the fact that the titre rises with extraordinary rapidity; on the other hand, chronic "carriers" with sequestra would probably remain at much the same titre. Recovering cases with fibrous adhesions sometimes give a persistent suspicious reaction of the fifth or sixth type for some weeks; it is possible that the causal organism is persisting in some unsuspected focus in such cases, e.g., inside the fibrous adhesions, but we have not tested this hypothesis to date. We are strongly of the opinion that any reaction greater than i/10 indicates that the animal possesses specific pleuro-pneumonia complement-fixing antibody in its blood serum, and that it is therefore infected in some degree, has recently been infected, or is passing through a transient reaction.

(iv) *Summary.*

It is obvious from the foregoing that the complement-fixation test described herein is an extremely valuable aid to the diagnosis of pleuro-pneumonia.

* Note that in only 3 out of 2,062 sera from entirely negative animals were reactions of type 7 obtained: in all our experimental animals, which were originally 0/10, the type 7 reaction was significant.

Our results indicate that the reaction is absolutely specific and occurs with no other pathological or physiological condition or infection that we have studied, that it is very sensitive and can detect cases even before pulmonary involvement has occurred, and that it possesses an unusual degree of reliability. In the acute stage, even when the disease is so mild that a clinical diagnosis is impossible, it is probably entirely accurate. At least in our hands, 100 per cent. of cases killed in the acute stage (25 out of 25) gave strong complement-fixation reactions; three other cases that showed resolving lesions of pleuro-pneumonia, from which the organism was cultivated, gave strong reactions on the day of slaughter; 32 out of 33 which had given a positive reaction for a period, but which had recovered and subsequently given a negative reaction, showed signs of earlier acute pleuro-pneumonia when examined *post mortem* some weeks or months later, while the remaining animal, No. 72, which was not examined until four months after its last positive reaction, may reasonably be assumed to have recovered completely, although signs of earlier infection may have been overlooked.

In chronic cases containing one or more infective sequestra, it is also extremely reliable. A total of 48 cases diagnosed on pathological grounds as chronic pleuro-pneumonia with sequestrum formation were examined. The sequestra of three of these that gave negative reactions were shown to be bacteriologically sterile. The remaining 45 may therefore have included some sterile cases, but bacteriological investigation was not often possible. However, 42 of these gave positive reactions, and another, after giving a persistent positive reaction for nine months, became negative only after being used for another experiment entailing intradermal inoculation. Therefore, even if we assume that the sequestra in the remaining three that were negative reactors contained viable organisms, there was still the very high correlation of 42 out of 45, i.e., 93.9 per cent. $\pm$ 3.65 per cent.; but, if we allow that one or more of the three might not have contained viable pleuro-pneumonia organisms, then the correlation is still higher.

V. DISCUSSION ON THE USE OF THE COMPLEMENT-FIXATION REACTION IN THE CONTROL OF PLEURO-PNEUMONIA.

1. General.

Firstly, consider a previously unvaccinated herd in which acute pleuro-pneumonia exists. As a result of our experience, we can expect the test to detect unerringly all animals with the pulmonary lesions, and even many cases in which these have not developed but in which infection is revealed only by enlarged oedematous mediastinal lymph glands. In addition, we might expect a small proportion of transient reactions. In our experience, out of 65 cattle exposed to continuous infection there were 4 such reactions of "positive" titre (iv/10), five of "weak positive" titre (iii/10), and four of "very weak positive" titre (ii/10); but it must be remembered that these instances were accumulated over two years of routine weekly testing. It is probable that, at any given moment during the progress of an outbreak in the field, transient reactions would be only rarely encountered. Unfortunately, we cannot yet say definitely whether post-mortem examination of such animals would reveal early lesions or at least yield cultures of the organism. However, our experience indicated that the majority

of such "transient reactors" are susceptible and would develop the disease in due course if allowed to continue in the infected environment. In other words, in an uninoculated herd in which acute pleuro-penumonia is occurring, usually only those animals that subsequently develop pleuro-pneumonia give transient reactions of full "positive" significance (iv/10).

Secondly, consider a herd that has been inoculated in the tail in the usual manner not later than eight weeks previously, and let us imagine them brought into contact with active cases of pleuro-pneumonia. Most of the vaccinated animals should be immune, but, as shown in Table 9, a proportion (in our experience, two out of nine) may prove susceptible and develop the disease in a mild form. Some may exhibit transient "positive" reactions (iv/10), but, in the limited number examined by us, this did not occur, whereas among seven resistant tail-vaccinated animals one gave a "positive" transient reaction. The probability is that there would be no essential difference in the proportion of "positive" transient reactions in either susceptible or resistant tail-vaccinated animals.

It follows, therefore, that there may be a certain proportion of deceptive transient reactions of "positive" titre amongst tail-vaccinated, as well as amongst unvaccinated animals exposed to infection. There appear to be two ways of avoiding these "false reactions" under such conditions; (*a*) by titrating the sera to their limits, when in general the transient reactions will not extend past iv/10, whereas sub-clinical cases of acute pleuro-pneumonia may go to iv/100, iv/200, or even iv/640; (*b*) where a re-test can be carried out on sera taken after an interval of a week, the differentiation should be sharper, for the transient reaction will probably have disappeared or will at most be stationary, but with the serum of an animal developing the disease the titre will have greatly increased. In the case of carriers, the reaction will be essentially the same as that obtained previously. When it is impossible to isolate "suspect" cattle for observation or very difficult to arrange re-tests, and when administrative action must be based upon a minimum of tests, the reactions must be interpreted very severely and all those greater than i/10 classed as positive.

There are two methods of controlling pleuro-pneumonia by the aid of the complement-fixation test, the first without the help of prophylactic vaccination, the second with it. The disadvantages of the first method are the serious infections and even deaths that occasionally follow tail vaccination, especially of dairy cattle, with virulent "natural virus" or culture, and the unavoidable vitiation of the complement-fixation test for about eight weeks after inoculation; the advantage is the relative protection conferred.

The choice of method must be determined by circumstances, depending upon whether we are dealing with dairy herds or pastoral properties. In Australia, particularly in Queensland, the Northern Territory, and the northern portion of Western Australia, pastoral properties are relatively enormous. Many have an area of 1,000 square miles, some even 10,000 square miles. Even though the carrying capacity may be low (from 5 to 15 per square mile), nevertheless the herds are large and may reach 100,000 head. These very large properties are always devoid of boundary fences and are not subdivided into paddocks. The staff is always small and the cattle half-wild, being rarely handled more

than once or twice in a year; a complete muster is practically impossible. During the annual mustering, counts are made and the natural increase deduced, calves are castrated and branded, and the cattle for sale are segregated. Then begins a long walk by road for perhaps many hundreds of miles, entailing possibly two or three months, the unfenced stock route passing through intermediate properties, until eventually the markets or abattoirs are reached. It will be readily realized that disease control under such conditions may have to be greatly modified, if it is to be practicable, and will be different from that adopted for small dairy herds in closely settled areas. The four main sets of circumstances may now be discussed.

2. Dairy Herds.

These are usually small and fairly easily protected from re-infection. Suspected reactors may usually be isolated, and adequate daily inspection both by the owner and Government officers is possible. Under such conditions, prophylactic vaccination may be dispensed with and the possibility of excessive tail reactions thereby obviated. The following procedure should be adopted (Method A):—

(*a*) Quarantine of the infected property and those in contact.

(*b*) Slaughter of clinical cases.

(*c*) Complement-fixation test of the remainder with immediate slaughter of reactors of types 1 to 5; reactors of types 6 and 7 to be isolated and re-tested one or two weeks later, or if isolation is not possible, to be treated as infected.

(*d*) Regular inspection and clinical examination (body temperature), to aid in the detection of cases while awaiting the result of blood tests.

(*e*) Repetition of test at weekly intervals until two consecutive completely negative tests are obtained.

(*f*) Maintenance of quarantine for the desired period* after the last active case.

(*g*) Obtaining of a completely negative herd reaction at the end of the quarantine period.

It will be realized that the above plan, by removing active sources of contagion early, will considerably shorten the quarantine period and, at the same time, by removing the potentially dangerous "chronic carriers", make the herd safe for the introduction of new members.

If the difficulties of efficient quarantine and lack of veterinary supervision make it desirable to utilize the known protective value of vaccination, the procedure should be modified as follows (Method B):—

(*a*) As for A.

(*b*) As for A.

(*c*) Collection of sera for testing, and simultaneous vaccination.

(*d*) On receipt of the results of the test, positives to be slaughtered (because vaccination is not known to have any curative effect upon already-existing pleuropneumonia either acute or chronic) and suspicious reactors either isolated for observation and re-test or treated as positive.

* The quarantine period varies: in Queensland it is usually 60 days, in Victoria 10 to 12 weeks, and in South Australia at least three months, after the last active case.

(*e*) As vaccination vitiates the interpretation of the reaction for a period of eight weeks, the animals should merely be placed under general supervision. Not earlier than eight weeks after vaccination (unless the tail reactions have been excessive, with invasion of the gluteal muscles, in which case the period should be extended for those animals) the herd should be re-tested and any "positives" removed for slaughter; weaker reactors should be either isolated for re-testing a week later or treated as positive.

(*f*) If no reactors are found at this test and another carried out a week or more later, the herd may be released from quarantine, providing the approved period from the last active case has elapsed. If reactors are found, they must be removed and re-tests carried out until two completely negative tests are obtained.

3. Comparatively Large Pastoral Properties.

A little more difficult to control are herds on the larger cattle-fattening or breeding properties that are nevertheless fairly well subdivided. Under these conditions, the number of tests must be reduced to a minimum; daily clinical examination may not be easily practicable; generally speaking, it will be preferable to vaccinate the herd, and thus, by the resulting immunization, compensate for any departure from the ideal serological control. The most suitable procedure would be as follows (Method C):—

(*a*) As for A.

(*b*) As for A.

(*c*) As for B.

(*d*) As for B, but weaker reactors will be treated as positive.

(*e*) As for B, but weaker reactors will be treated as positive.

(*f*) Await the termination of the quarantine period.

In this case, the complement-fixation test, by enabling early detection of infected animals, will have reduced the losses within the herd, and by the detection of the chronic carriers will remove the danger of the herd's subsequently spreading infection. Even if reactors are occasionally missed, through reduction in the number of tests, we shall still be in a much better position than without it, under which circumstance no attempt can be made to eliminate them.

4. Very Large Unfenced Pastoral Properties.

When we are faced with the problem of the enormous unfenced properties typical of the great pastoral areas, it does not appear that the test can be practicably or very usefully applied, because the numbers of animals are too large. It is impossible to muster all of them, and impossible to guard them from contact with cattle on neighbouring properties or with travelling stock. All that can be done is to destroy any observed clinical cases and vaccinate the remainder.

5. Cattle to be Travelled.

The test could be of value under such circumstances. The present practice, at least in Queensland and the Northern Territory, is to vaccinate the mobs, which usually number about 1,000, before departure, but, of course, this has no effect on possible chronic carriers, which may spread the active disease later to inefficiently immunized members of the herd or to cattle in other herds. The slaughter of chronic carriers before departure of the mob would remove this danger. A reasonably satisfactory plan, considering the great practical difficulties, would be the following (Method D):—

(*a*) Cattle intended for travelling to be segregated in an isolated part of the property.

(*b*) Any obvious clinical cases to be destroyed.

(*d*) Collection of sera and vaccination to be carried out simultaneously.

(*e*) On receipt of the result of the test, reactors to be destroyed and the remainder to begin travelling.

The chances of missing a sub-clinical or chronic case will be very low, provided the system of identification of animals and samples is efficient, and vaccination should reduce to negligible proportions the possibility of an outbreak through the accidental inclusion of an infected animal. Such a procedure, reinforced by a second test when the cattle reach their destination some months later, should remove the possibility of the introduction of reactors into the new environment, and, with adequate departmental veterinary control and supervision, should make possible the comparatively safe introduction of cattle from the infected northern properties to the uninfected fattening properties in the south, and thus remove a very great disability under which many northern pastoralists suffer, viz., the total prohibition of the sending of their cattle into non-infected areas, where the main markets lie. Even if by some mischance of technique a very occasional outbreak of pleuro-pneumonia should result in the previously non-infected herds, the use of the complement-fixation test with adequate veterinary staff would make its speedy control possible.

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Appendix.

SYNOPSIS OF CATTLE IN THE NATURAL-INFECTION HERD THAT CONTRACTED PLEURO-PNEUMONIA.

No.	Incubation Period (Days).	Clinical Report.	Interval Between Appearance of Antibody and Clinical Symptoms.	Maximal Titre.	Interval During which Antibody Detectable in Blood.	Interval Between Clinical Diagnosis and Death (D) or Slaughter (S).	Titre at Death.
				(A) AUTOPSIED AT THE ACUTE STAGE.			
				(i) *Unvaccinated.*			
18	41	Very acute	0	iv/$\overline{200}$ ..	Until death	(D) 3	iv/$\overline{200}$
21	124	„ „	0	iv/380, iii/$\overline{400}$	„ „	(D) 13	iv/380, iii/$\overline{400}$
74	93	Hyperacute	1	iv/160, iii/$\overline{200}$	„ „	(D) 10	iv/160, iii/$\overline{200}$
80	148	„	2	iv/$\overline{440}$ ..	„ „	(D) 20	iv/$\overline{440}$
83	85	„	11	iv/440 ..	„ „	(S) 5	iv/$\overline{440}$
91	95	„	12	iv/$\overline{200}$ ..	„ „	(D) 15	iv/$\overline{200}$
140	Not known	Acute ..	Not known	iv/$\overline{200}$ ..	> 17	(D) > 17	iv/$\overline{200}$
150	„ „	Hyperacute	„ „	iv/$\overline{100}$ ..	> 5	(D) > 5	iv/$\overline{100}$
164	„ „	„	„ „	iv/$\overline{200}$ ..	> 3	(D) > 3	iv/200
181	„ „	„	„ „	iv/$\overline{200}$ ..	> 3	(S) > 3	iv/$\overline{200}$
234	261 (174)	Very mild	1	iv/$\overline{200}$ ..	Until death	(S) 1	iv/$\overline{200}$
177	Not known	Hyperacute	− 2	iv/$\overline{200}$ ..	„ „	(D) 8	iv/$\overline{200}$
241	258 (164)	Mild ..	6	iv/$\overline{200}$ ..	„ „	(S) 3	iv/$\overline{200}$
264	180 (143)	Hyperacute	14	iv/200 ..	„ „	(S) 1	iv/200
265	159 (122)	Subacute..	0	iv/40, i/100..	„ „	(S) 3	iv/40, i/100
269	141 (104)	Very mild	− 1	iv/40, i/100..	„ „	(S) 4	iv/40, i/100
272	141 (104)	„ „	6	iv/60, i/100..	„ „	(S) 4	iv/60, i/100
277	Not known	Hyperacute	Not known	iv/$\overline{200}$ ..	> 3	(D) > 3	iv/$\overline{200}$
281	60	Masked ..	5	iv/$\overline{40}$, i/100..	Until death	(D) 0	iv/40, i/100
288	139	Acute ..	3	iv/160, i/200	„ „	(S) 6	iv/160, i/200
289	131	„ ..	9	iv/100, i/180	„ „	(D) 0	iv/100, i/180
291	119	Subacute..	4	iv/100, ii/30	„ „	(S) 5	iv/10, ii/30
294	Not known	Hyperacute	Not known	iv/$\overline{200}$ ..	„ „	(D) 8	iv/$\overline{200}$
307	„ „	Acute ..	„ „	iv/20, i/60 ..	„ „	Not known	iv/20, i/60
				(ii) *Vaccinated.* None.			
				(B) AUTOPSIED IN STAGE OF RESOLUTION.			
				(i) *Unvaccinated.*			
235	98	Subacute..	9	iv/$\overline{200}$ ..	121	(S) 112	iv/10, ii/30
405	Not known	Hyperacute	Not known	iv/$\overline{200}$ ..	Until death	(S) > 26	iv/$\overline{200}$
170	„ „	Acute ..	„ „	iv/$\overline{200}$ ..	„ „	(S) > 27	iv/$\overline{200}$
				(ii) *Vaccinated.* None.			

APPENDIX—*continued*.

No.	Incubation Period (Days).	Clinical Report.	Interval Between Appearance of Antibody and Clinical Symptoms.	Maximal Titre.	Interval During which Antibody Detectable in Blood.	Interval Between Clinical Diagnosis and Death (D) or Slaughter (S).	Titre at Death.
(C) Autopsied at Chronic Stage (Sequestra Containing Viable Organisms).							
(i) *Unvaccinated.*							
22	152	Acute	2	iv/$\overline{200}$	Until death	(S) 159	iv/20, iii/$\overline{30}$
89	200	Subacute	19	iv/200	,, ,,	(S) 148	iv/40, i/100
168	Not known	Acute	2	iv/$\overline{200}$	,, ,,	(S) 78	iv/100, i/180
193	327 (147)	Mild	4	iv/$\overline{200}$	,, ,,	(S) 117	iv/10, ii/30
282	134	,,	− 2	iv/100, i/160	,, ,,	(S) 36	iv/60, i/120
303	Not known	Acute	Not known	iv/$\overline{200}$	,, ,,	(S) > 34	iv/20, i/80
308	,, ,,	Not known	,, ,,	iv/$\overline{200}$	> 358	(S) > 310	o/10
166	,, ,,	Mild	0	iv/40, i/100	12	(S) 12	iv/40, i/100
(ii) *Vaccinated.*							
31	48	Subacute	7	iv/$\overline{200}$	Until death	(S) 210	iii/11, i/30
(D) Autopsied in the Healed Stage (including Sterile Sequestra Marked "*").							
(i) *Unvaccinated.*							
1	29	Very mild	1	iv/$\overline{30}$	28	(S) 196	o/10
11	117	Mild	2	iv/$\overline{200}$	60	(S) 196	o/10
23	115	,,	0	iv/$\overline{200}$	104	(S) 202	o/10
25	105	Subacute	− 2	iv/$\overline{200}$	140	(S) 212	o/10
35	79	Mild	20	iv/200	202	(S) 240	o/10
39	79	Acute	15	iv/$\overline{200}$	138	(S) 196	o/10
72	125	Very mild	0	iv/80, i/160	21	(D) 151	o/10
73	153	,, ,,	0	iv/60, i/100	76	(S) 195	o/10
76	155	Acute	16	iv/$\overline{200}$	125	(S) 182	o/10
85 (*a*)	127	Mild	− 4	iv/40, i/100	26	..	..
85 (*b*)	34	Acute	3	iv/60, i/120	87	(S) 143	o/10
94	96	Mild	39	iv/200	116	(D) 77	iii/10
225	154 (67)	Very mild	0	iv/10, ii/$\overline{30}$	21	(S) 253	o/10
227	217 (130)	Mild	7	iv/$\overline{200}$	175	(S) 171	o/10
233	122 (86)	,,	5	iv/140, ii/$\overline{200}$	168	(S) 226	o/10
238	161 (74)	Acute	0	iv/180, iii/$\overline{200}$	152	(S) 238	o/10
255	243 (207)	Very mild	13	iv/$\overline{200}$	83	(S) 92	o/10
267	201 (165)	,, ,,	14	iv/120, i/160	57	(S) 135	o/10
270*	117 (81)	,, ,,	7	iv/60, i/120	175	(S) 227	o/10
275	Not known	Mild	Not known	iv/$\overline{200}$	> 49	(S) 245	o/10
280	160 (124)	Very mild	14	iv/100, i/160	98	(S) 148	o/10
283	138	Mild	6	iv/140, i/180	114	(S) 162	o/10
284	116	,,	− 2	iv/140, ii/$\overline{200}$	126	(S) 192	o/10
285	139	Very mild	Not known	iv/10, ii/$\overline{30}$	42	(S) 139	o/10
286	154	Mild	8	iv/160, i/200	63	(S) 149	o/10
290	124	,,	− 1	iv/$\overline{200}$	147	(S) 172	o/10

APPENDIX—*continued.*

No.	Incubation Period (Days).	Clinical Report.	Interval Between Appearance of Antibody and Clinical Symptoms.	Maximal Titre.	Interval During which Antibody Detectable in Blood.	Interval Between Clinical Diagnosis and Death (D) or Slaughter (S).	Titre at Death.
(D) AUTOPSIED IN THE HEALED STAGE (INCLUDING STERILE SEQUESTRA)—*continued.*							
(i) *Unvaccinated.*							
292	195	Very mild	Not known	iv/20, iii/$\overline{30}$..	17	(S) 115	o/10
296	139	,, ,,	,, ,,	iv/20, i/80 ..	35	(S) 171	o/10
305	Not known	Mild ..	,, ,,	iv/10, ii/30 ..	> 28	> (S) 231	o/10
192	215 (56)	Very mild	,, ,,	iv/20, i/60 ..	36	(S) 241	o/10
236	123 (87)	Mild ..	21	iv/40, i/120 ..	169	(S) 205	o/10
(ii) *Vaccinated.*							
2	127	Mild ..	− 4	iv/$\overline{200}$ ..	62	(S) 88	o/10
61	251	,, ..	6	iv/$\overline{200}$ ..	89	(S) 118	o/10
70	92	Very mild	7	iv/$\overline{30}$ ..	21	(D) 52	o/10
244	103	Mild ..	1	iv/10, ii/30 ..	49	(S) 265	o/10

NOTES.—(*a*) No. 85 underwent two attacks of pleuro-pneumonia, the second more severe than the first.

(*b*) The incubation period is calculated from the day of entry into the infected environment until the day on which clinical symptoms developed; when these were absent the day on which the first iv/10 complement-fixation reaction occurred is taken.

As the animals were in continued contact, the incubation period is in each instance the maximum; actually it might have been less, for under those conditions it is not possible to determine when the infection occurred. The incubation period within brackets is calculated from the introduction of the second batch of acute cases and is given for those animals that resisted the first outbreak, but became infected during the second. In the case of animals that were brought to the laboratory already infected, the incubation period is not known (n.k.).

(*c*) Sometimes the disease was so mild that no clinical signs were apparent at any period; for these animals and certain others that were recovering when acquired by us the abbreviation "n.k." (not known) is used.

(*d*) As clinical examinations were carried out daily, whereas serological testing was performed only weekly, the figures in column four are approximate and apparent only; they might in some cases have to be increased by as much as six days, thus converting apparent negative figures into positives.

(*e*) In the case of animals already clinically affected when acquired by us, only minimum values can be assigned to the periods in columns six and seven.

(*f*) In addition to the 70 susceptible animals described in the above synopsis there were sixteen unvaccinated (including five "transient reactors") and fourteen vaccinated animals including six "transient reactors") that completely resisted the infection over periods as long as 446 days. They showed no clinical signs, no complement-fixation reactions (excepting the transient reactions among those mentioned), and at autopsy were completely normal, with no indications of recovery from earlier infection.

II(a). Observations on the Diagnosis of Bovine Contagious Pleuro-pneumonia by Means of the Complement-fixation Test of Campbell and Turner.

*By H. R. Seddon, D.V.Sc.**

1. General.

On account of the difficulty of clinically detecting animals affected with chronic lesions of contagious pleuro-pneumonia, i.e., animals with lung sequestra, often containing the virus, and hence "carriers", the applicability of a biological test for diagnosis has constantly exercised our minds, and methods which have been elaborated by others have been tested with a view to their utilization. Both complement-fixation and agglutination tests were described by Heslop (1 and 2). The technique of the former was such that it did not seem generally applicable in routine testing, and in our hands the latter was unreliable and therefore was discarded.

Later, following Gregory's (3) application of the complement-fixation test according to the technique of Tietze and Giese, Hindmarsh (4), of this Station, employed it with remarkable success in dealing with two outbreaks of the disease in New South Wales.

Stamping-out by slaughter was practised, and all animals showing any degree of fixation (though with no clinical sign of contagious pleuro-pneumonia) were killed, with the following results:—

Reaction.	Number.	Found Affected with C.P.P.
Positive	8	8
Highly suspicious	2	2
Suspicious	2	1

Thereafter, the herd was kept under surveillance, and, as four years have now elapsed and no recrudescence of the disease has occurred, it is believed that, through the detection of affected animals by the complement-fixation test, a feat not possible clinically, the disease was effectually stamped out.

Whilst these results were good indeed, the technical difficulties attendant on the test were such that it was not considered to be a practicable procedure for utilization in a routine fashion, or for dealing expeditiously with a large number of animals.

* Director of Veterinary Research, Glenfield Research Station of the New South Wales Department of Agriculture.

In 1933, the writer had the opportunity of gathering, at first hand, a knowledge of the (then) newly elaborated technique of Campbell and Turner (5), and, thanks to their courtesy, a knowledge of the results they had then attained. It was decided, therefore, to test it out at Glenfield.

Unfortunately, we were not able to secure any large quantity of "virus" from which to prepare antigen, and that obtained has never, in our hands, furnished an antigen of high titre. Thanks to the kindness of Dr. Turner, however, we have been supplied by him with ample antigen, and this has been used in all our tests.

In the test, we depart slightly from the technique of Campbell and Turner, but such is only in manipulative procedure—whereas they leave 0.5 ml. of serum dilutions of 1 in 10, 1 in 20, and 1 in 30, respectively, in their tubes, we add 0.5 ml., 0.33 ml., and 0.16 ml. of a 1 in 10 dilution. The total saline content is made up to 2 ml. in each tube, so that, in our tubes, the serum is diluted to the same extent as by them.

Our tubes therefore contain:—

Ingredient.	Actual Test.			Control.
Serum	0·05 ml.	0·033	0·016	0·05 ml.
Antigen	5 units	5 units	5 units	..
Complement	2½ units	2½ units	2½ units	2½ units
Red cells 3 per cent. suspensions ..	0·5 ml.	0·5 ml.	0·5 ml.	0·5 ml.
Haemolytic serum..	3 MHD	3 MHD	3 MHD	3 MHD

We have recorded our results in the same fashion as Campbell and Turner, the degree of fixation being estimated 1, 2, 3, or 4, the figure 1 representing slight, and 4 complete, fixation. At times, just a trace of fixation, manifested by a mere smokiness, is seen in the first tube. This we designate a "trace" (tr.), and do not consider as "definite recognizable evidence of fixation".

In our records, complete fixation in all three tubes of the actual test is represented as a 4.4.4. reaction, and the absence of fixation in any as 0.0.0., the first figure referring to the tube containing 0.05 ml. serum, and the last to that having 0.016 ml.

2. Tests on Animals which we had every reason to believe were free from the Disease.

(i) *Our Experimental Herd at Glenfield.*

This herd has now been maintained for fourteen years, and has been built up chiefly by natural increase. Though cattle have been introduced from outside, they have almost invariably been animals which were under one year of age at time of purchase, and have been from herds known personally to us, and which we have every reason to believe have not suffered from contagious pleuro-pneumonia.

These animals, 64 in number, have been subjected to the complement-fixation test, many being re-tested, and all have given completely negative reactions (0.0.0.).

(ii) *Cattle from Pleuro-free Districts.*

We have, at times, included in our tests certain sera sent in for agglutination test for contagious abortion, particularly in trying out a new batch of antigen, such sera being selected from herds in "pleuro-free districts". (These are parts of the State maintained free from contagious pleuro-pneumonia by restriction of import therein to cattle which have not had contact with cases of the disease, and then only after inspection). These sera have likewise given completely negative (0.0.0.) tests. Such sera would number about twenty.

(iii) *Cattle Intended for Export.*

These were animals from herds not affected with contagious pleuro-pneumonia, and were all pedigreed bulls. The test was applied under requirements of the importing country.

To date some nineteen such animals have been tested, and all gave completely negative reactions (0.0.0.), except that two gave a trace of fixation (less than 1) with 0.05 ml. serum, none with 0.03 or less (i.e., tr. 0.0 reactions).

Summary of animals from herds unaffected with contagious pleuro-pneumonia.

Tested.	Reaction 0.0.0.	Reaction tr. 0.0.
103	101	2

3. Tests on Animals Affected with Contagious Pleuro-pneumonia.

These animals fall into two groups, viz.:—

(*a*) Clinical cases.

(*b*) Detected by complement-fixation test.

(i) *Clinical Cases.*

In response to our request, Stock Inspectors furnished sera from three cases of contagious pleuro-pneumonia killed when in the acute stage of the disease, whilst three clinical cases (detected by test some days before symptoms developed and brought to the Station) are also included here. All of these six animals gave complete fixation in the three tubes of the test, and the reactions are therefore recorded as 4.4.4. reactions.

(ii) *Detected by Complement-fixation Test.*

Some 28 animals not showing symptoms of contagious pleuro-pneumonia, and hence cases which could not be diagnosed clinically, have been the subject of post-mortem examination, and correlation of the pathological findings with the test is therefore possible (see appendix). The value of this comparison is discounted to some extent, however, by the delay which has often occurred between testing and destruction.

These animals came from two herds:—In the *first herd* the test was utilized as a means of picking out all possible infected animals, and all that gave a reaction in excess of a 1.0.0. reaction were destroyed. The

three animals in this herd which gave 1.0.0. reactions were not destroyed, as they came from portions of the herd in paddocks distant from the infected mob, and, no definite reactors being detected in their mob, it was considered that the marked difference in intensity of reaction as given by them, and by animals found to be chronic cases of contagious pleuro-pneumonia, was sufficient to regard the three in question as being non-infected. In the *second herd,* from which these reactors were obtained, destructions, apart from some twelve destroyed by us, were at the discretion of the owner, and some animals in this herd showed 2.1.0. reactions. Unfortunately, post-mortem examination of these was not possible.

As we know that the reactions given by an animal may vary in the course of a few days, in considering the table, prime importance is attachable to those animals killed on the day of test, or within a day or two. Fortunately, animals showing reactions of 3.1.1. to 4.4.4. were destroyed on the day of test, and it is important to note that we have an instance of lesions no greater than a sequestrum 2″ x 1″ x 1″ (size of large walnut or small egg) given by animals exhibiting respectively, 4.4.4.; 4.4.3.; and 3.1.1. reactions on day of test. Whilst nearly all of the 4.4.4. animals in the table showed only sequestra on post-mortem examination, the fact that in many cases considerable intervals (24 to 51 days) had often elapsed suggests that, when tested, they may have had more extensive lesions, and the fact that non-clinical cases may actually have acute lesions (*vide,* Nos. 5, 131, and 74) supports this. Nevertheless, the results of 2, 70W, 48, 49, and 138 show that the lesions may, even then, have been chronic in nature. Some information bearing on the question of resolution is given later.

Consideration of the Significance of the Reactions Obtained.— Whilst we have not had the opportunity of undertaking a post-mortem examination on an animal then testing 1.0.0., we are of opinion that such is so close to a tr.0.0. reaction, even to a 0.0.0. reaction, that it does not necessarily indicate infection with contagious pleuro-pneumonia and is compatible with absence of infection. Greater reactions, such as 2.1.0. and 2.1.1., have been met with only in a herd where the disease occurred, and we think should be regarded as specific reactions. They are possibly too weak to warrant destruction, but should certainly be regarded as "suspicious" and re-tested. If they then give a greater reaction, the proper course of action is clearly indicated. If the reaction falls, then they would appear to be recovering cases, similar to those to be described later. We consider, therefore, that evidence of pleuro-pneumonia is furnished only when there is some degree of fixation in the second tube of the test.

In summary we find, therefore, that animals may give reactions as follows:—

1. Acute lesions only .. .. 4.4.4.
2. Acute with sequestra .. .. 4.4.4.
3. Sequestra only—
   - (i) Large .. .. .. 4.4.4., 4.4.3., or 3.2.1.
   - (ii) Small .. .. .. 4.4.4., 4.4.3., or 3.1.1.
4. Probably infected .. .. 2.1.1. and 2.1.0.
5. Probably non-infected .. .. 1.0.0.
6. No lesions .. .. .. 0.0.0. and tr.0.0.

4. Progressive Reactions in Contagious Pleuro-pneumonia.

Though our opportunities of making observations in this regard have been limited, information is forthcoming concerning three negative animals which later reacted, and two reactors in which all trace of reaction subsequently disappeared. Interest attaches to these cases, as they furnish some evidence as to the time taken for lesions to develop and to retrogress. Details of these cases are as follows:—

No. 40.—A cow brought to this Station in a motor lorry, along with two clinical cases of contagious pleuro-pneumonia. These clinical cases were killed the same day, lesions of acute contagious pleuro-pneumonia being found present in the lung.

Cow No. 40 was then isolated, and, as it later developed contagious pleuro-pneumonia, it evidently contracted the infection either when running in the herd or during transit. As its last known contact with an *active* case was the day of receipt, that date will be taken in discussing the period of incubation and recording the course of the disease.

Complement-fixation tests on date of receipt, and on the thirteenth and twenty-fourth days afterwards were all completely negative. On the twenty-fifth day, the animal showed a rise of temperature, the febrile reaction subsiding after three days.

Complement-fixation tests on the twenty-seventh and thirty-fourth days were still completely negative. On the thirty-sixth day, the animal's temperature again rose, and remained high till the forty-fifth day, a temperature of 106° being recorded that day. Complement-fixation tests during this febrile period were suspicious (2.1.0.) on the thirty-seventh, and positive on the forty-first and forty-fourth days (4.3.3. and 4.4.3 respectively). At the time of these last two tests, the animal exhibited a cough. It aborted on the forty-seventh day, a strong positive (4.4.4.) being given that day. Thereafter, there was intermittent fever, coughing was persistent, and the animal lost considerably in condition. A complement-fixation test on the fifty-first day was strongly positive (4.4.4.). On the fifty-eighth day, there was a putrid nasal discharge, and the animal's appetite became depressed, food later being refused entirely. By the sixty-second day, the animal was very weak, and it was therefore destroyed. (Complement-fixation reaction 4.4.4.).

Post-mortem examination showed a large encapsuled lesion of contagious pleuro-pneumonia in the left lung. The cavity containing the sequestrum was septic, and the putrid nasal discharge, noted during the previous week, had evidently come from it, the cavity communicating with a bronchus. The fibrous tissue around the lesion and between the overlying pleural surfaces was evidently recent. There is thus every reason for believing that the lesions seen were from infection contracted on the day of receipt of the animal here, or slightly before.

Summarized, this animal showed—

(*a*) A transient rise of temperature from the twenty-fifth to twenty-eighth days.

(*b*) Completely negative reaction up till thirty-four days after infection.

(*c*) A suspicion reaction on the thirty-seventh day.

(*d*) Continued febrile attack from the thirty-sixth day, rising to maximum on the forty-fifth day.

(*e*) First definite positive reaction on the forty-first day.

(*f*) Cough from the forty-fifth day.

(*g*) Sequestrum found on post-mortem examination on the sixty-second day.

If it is concluded, as seems reasonable, that acute lesions were first present not earlier than the fortieth day, then it will be seen that sequestrum of an acute lesion may develop in about 22 days. The incubation period, as deduced by complement-fixation reaction, would be about 40 days.

No. 72.—Information regarding this animal is very scanty. A cow testing tr.0.0. was killed 23 days later, being sent to the boiling-down works by the owner, along with a number of animals which we had previously classed as reactors. Whether it was showing symptoms or not we do not know, but interest lies in the fact that the Inspector who made a post-mortem examination found the whole of the right lung affected with lesions of contagious pleuro-pneumonia in an acute form.

It is evident that an animal which on test would be classed as negative may show acute lesions in 23 days.

No. 70*W*.—This animal was tested on only three occasions. The first test was completely negative (0.0.0). Tested 35 days later, it was strongly positive (4.4.4), subsiding slightly (to 4.4.3) by the 47th day, by which time the only lesion present was a necrotic sequestrum the size of a walnut.

It is evident, therefore, that within a period of 47 days an animal giving a negative reaction may suffer acute contagious pleuro-pneumonia, and the lesions (probably not extensive) retrogress to small encapsuled areas.

No. 10.—At its original test, this animal gave a 4.4.4 reaction. It was showing no clinical signs of the disease, but was brought to the Station, along with a number of other reactors, for slaughter. At the time of its receipt, 24 days after initial test, it gave a 3.3.2 reaction, and in view of the diminished reaction it was held for observation. Re-tested on the 58th day, it gave a completely negative (0.0.0) reaction, and the same on the 85th day.

It was destroyed 120 days after the first test, this being over two months since the first negative test recorded.

Post-mortem examination showed adhesion of the posterior lobe of the left lung to the chest wall, both pleurae, especially the costal, being thickened, the adhesions being tough and covering an area approximately 5 inches by 4 inches.

On breaking down the adhesions, a definite depression was found near the edge of the lobe and palpation showed lumpiness or cording at the base of this pit. Incision of the "corded" mass showed it to consist of greyish fibrous tissue with no sign of necrosis.

Evidently, therefore, this animal had completely recovered, and the pit, or depression, marked the site of a lesion, probably originally of

acute pneumonia that later became necrotic, and which was finally absorbed, with consequent atrophy of lung substance, its part being to some extent replaced by the fibrous (scar) tissue felt on palpation.

No. 135.—This animal was from the same herd as No. 10 and was a reactor brought to the Station for slaughter. At its initial test it gave a reaction recorded as 3.3.3. On arrival at the Station, 17 days later, its reaction had declined to 2.1.1. Re-tested on the 57th day, it gave a complete negative (0.0.0), and the same on the 84th day. It was killed on the 109th day after first test, its first negative having been recorded 42 days previously.

Post-mortem examination showed the inner aspect of the left posterior lobe to be adherent to the diaphragm for an area of about 1½ inches in diameter. On severing the adhesion, a definite pit, about 1 inch in diameter and ½ inch in depth, was found in the centre of the adherent area. At the base of the pit was dense fibrous tissue with some small necrotic masses embedded in it. The middle third of the middle lobe on the left side was much fibrosed and adherent to the ribs, but no necrotic areas were detected.

It seems evident that the very small areas of necrotic tissue under the adhesion in the posterior lobe represented the sequestrum almost completely resolved, and the depression, atrophy of pulmonary tissue in that area.

Comment on Cases Nos. 10 *and* 135.

Two features of these cases may be discussed—

First, they are cases in which there were present *reparative* processes compatible with the previous existence of lesions of contagious pleuro-pneumonia.

Second, they show that, whilst a positive reaction is given by animals showing necrotic sequestra of the size of a walnut, the animal may recede to a completely negative test whilst there is some, albeit small, necrotic tissue still left in the lung. The observations on these cases suggest that, when the virus in a sequestrum is destroyed, the absence of the antigen responsible leads to very rapid elimination of complement-fixing antibodies from the blood.

5. Application of Complement-fixation Test in Eradication of Contagious Pleuro-pneumonia from a Herd.

On a large property in the Northern Tablelands, three animals were reported to the District Veterinary Officer as having recently died, and another animal of the same mob killed by him on 7th August, 1934, was found to show lesions of contagious pleuro-pneumonia in the acute form.

The herd, together with contacts on the property, totalled 633, all cows, whilst 68 animals on adjoining holdings and 3 suspected animals, earlier contacts with part of the above but then in another district, were also tested.

The animals on this property were divided into several "mobs" according to their paddocking and their origin. A complete test of all animals was made and, depending on the result of this, an additional

test as thought necessary. All animals giving a reaction greater than 1.0.0 were regarded as reactors. The results of these tests were as follows: —

Mob (*a*), 57 animals. (This was the mob in which four clinical cases had been found.)

First Test.—Three reactors found. Killed 16 days after test. All showed necrotic lesions of contagious pleuro-pneumonia on post-mortem examination.

Second Test.—Applied 39 days after first test. No reactors found.

Mob (*b*), 93 animals. Contacts with the above.

First Test.—Two reactors. Killed 16 days after test. One showed a chronic and an acute lesion of contagious pleuro-pneumonia (apparently a case of auto-supra-infection), and the other a chronic lesion.

Second Test.—Applied 35 days after first test. One reactor found. Killed 13 days after second test. Showed a necrotic lesion of contagious pleuro-pneumonia.

Third Test.—Applied 34 days after second test. No reactors found.

Mob (*f*), 94 animals. (The only other mob to show a reactor).

First Test.—One reactor found. This animal was killed by the owner at time of bleeding as it was poor in condition and weak. No post-mortem examination was made.

Second Test.—Applied 37 days after first test. No reactors found.

Mobs (*c, d, e, g, h, i, j, k, l,* and *m*), 389 animals.

First Test.—All negative.

Second Test.—Undertaken of three only and not for any particular reason. No reactors found.

Mob (*n*), 3 animals. (Originally contacts with part of the above, but now in another district).

First Test.—No reactors.

Second Test.—Not undertaken.

Mobs (*o*) and (*p*), 68 animals on property adjoining.

First Test.—No reactors.

Second Test.—Not undertaken.

Comment.

The reactors mentioned above as killed are all included in the Appendix. They are animals Nos. 59B, 70W, 74, 105, 126, and 137. No. 59A, a non-reactor, was killed also, and on post-mortem examination showed no lesions of contagious pleuro-pneumonia.

Following destruction of reactors, no subsequent evidence of the disease was manifested, and, as some twelve months have now elapsed since the last reactor was destroyed, it is believed that the disease has been eradicated by the application of the measures adopted.

6. Discussion.

The results given in this paper, we feel, afford us every reason for believing that in the complement-fixation test, applied according to the technique of Campbell and Turner, we have a test of high reliability indeed. Moreover, it is one which, provided proper identification of animals is undertaken, can be performed on large numbers without technical difficulty, and for that reason should be of considerable value in dealing with contagious pleuro-pneumonia whether occurring epizootically or enzootically.

As animals which have been subjected to Willemsian vaccination may react to the complement-fixation test for some weeks after vaccination, such a practice cannot well be adopted in herds in which it is intended to obtain, by repeated testing, the full benefits to be gained from the test.

Whilst the chronically affected animal may be a carrier, it is evident that a percentage of these may, sooner or later, throw off the infection and recover. Before this, however, such an animal may possibly disseminate the disease. Stamping out by destruction of reactors may therefore entail the death of animals which might later have recovered; but, as we have frequently observed before, these chronically affected animals (carriers with a sequestrum) often light up, evidently from supra-infection as shown by the presence of acute lesions around a necrotic area, and at the time when the animal reacts it is quite impossible to foretell the course the infection will take.

Failure to detect and remove all infected animals, i.e., all reactors, would thus assist the disease to become enzootic in the herd. To eliminate it and keep it out, therefore, testing and re-testing, with testing of introduced animals, appears to be the rational method of controlling the disease. As in all such matters, however, economic considerations have considerable bearing, and for that reason the applicability may be chiefly in cases where the disease is epizootic, or where it is desired to introduce only clean cattle into an environment that is free from contagious pleuro-pneumonia. Its value where the disease is enzootic is undoubted, as it offers the only possible means of detecting carriers.

7. Acknowledgments.

It is desired to acknowledge the valuable technical assistance rendered by Mr. J. S. Freeman, Senior Laboratory Assistant at this Station, in conducting the complement-fixation tests recorded in this paper.

8. References to Literature.

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Appendix.

NON-CLINICAL CASES DETECTED ON COMPLEMENT-FIXATION TEST.

Animal.	Reaction.	Days between Test and Post-mortem Examination.	Result of Post-mortem Examination.
2	4.4.4	0	Chronic C.P.P. Sequestrum 3″ x 2″ x 2″
5	4.4.4	0	Acute C.P.P.
48	4.4.4	1	Chronic C.P.P. Sequestra 3″ x 3″ x 3″ and 2″ x 2″ x 2″. Pleuritic adhesions
49	4.4.4	1	Chronic C.P.P. Sequestrum 2″ x 2″ x 2″. Pleuritic adhesions
138	4.4.4	2	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″
50	4.4.4	5	Chronic C.P.P. Sequestrum 1½″ x 1½″ x 1½″
141	4.4.4	5	Acute C.P.P. Chronic and sub-acute lesion, half posterior lobe
26	4.4.4	6	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″
34	4.4.4	6	Chronic C.P.P. Sequestrum 4″ x 3″ x 2″
126	4.4.4	16	Chronic C.P.P. Sequestrum 4″ x 2″ x 1½″; also sub-acute lesion. Pleuritic adhesions
74	4.4.4	16	Acute C.P.P. Sequestrum 5″ x 3″ x 3″
91	4.4.4	24	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″
97	4.4.4	27	Chronic C.P.P. Sequestrum 6″ x 4″ x 3″
70	4.4.4	27	Chronic C.P.P. Sequestrum 3″ x 2″ x 1½″
19	4.4.4	44	Chronic C.P.P. Sequestrum 4″ x 3″ x 2″
28	4.4.4	44	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″
73	4.4.4	44	Chronic C.P.P. Sequestrum 4″ x 3″ x 2″
95	4.4.4	44	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″
56	4.4.4	48	Chronic C.P.P. Sequestrum 4″ x 3″ x 2″
58	4.4.4	51	Chronic C.P.P. Sequestrum 2″ x 2″ x 2″
70W	4.3.3	0	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″
47	4.4.3	48	Chronic C.P.P. Sequestrum 4″ x 3″ x 2″
137	4.3.3	16	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″
4	4.2.0	27	Acute C.P.P.
105	3.2.1	16	Chronic C.P.P. Sequestra 4″ x 2″ x 2″ and 3″ x 1½″ x 1½″
98	3.2.1	44	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″
80	3.2.1	70	No lesions C.P.P. (Meat Inspector)
59B	3.1.1	0	Chronic C.P.P. Sequestrum 2″ x 1″ x 1″

NOTE.—In many cases, only the affected portion of lung was submitted and pleuritic adhesions may have been present but not noted.

II(b). The Complement-fixation Test for Pleuro-pneumonia.

*By H. E. Albiston, D.V.Sc.**

The value of a sero-diagnostic test in the control of contagious bovine pleuro-pneumonia was clearly demonstrated in Victoria eight years ago, during an outbreak which followed the introduction of infected cattle from New South Wales. At that time, the complement-fixation test elaborated by Gregory from the technique of Tietze and Giese was used at this laboratory, and the discovery by this test of a number of "carriers" which otherwise would have escaped detection undoubtedly had a profound influence on the course of the outbreak. As a result of its employment, together with the effective administrative action adopted by the Veterinary Branch of the State Department of Agriculture, Victoria became free and remained free from infection for several years.

In spite of the good results obtained, especially with "carriers," this test was by no means infallible, and it possessed certain disadvantages which are adequately dealt with in this Bulletin by Campbell and Turner.

In November, 1933, a mob of 900 odd bullocks was introduced from a property in New South Wales on which an outbreak of pleuro-pneumonia had occurred in June of that year. After sale at Wangaratta, these animals were distributed to 14 properties in Victoria, and shortly afterwards pleuro-pneumonia made its appearance on 8 of them. On the occasion of this new outbreak, blood sera from over 800 bullocks were submitted to the complement-fixation test applied according to the technique of Campbell and Turner.

In December, the owner of one lot of 25 bullocks decided to have them slaughtered at an abattoir, and a preliminary test was conducted on the sera from these animals. One sample gave a strong positive reaction, and the beast from which the sample came was found on slaughter to show a resolving lesion of pleuro-pneumonia in one lung. All other samples were negative to the test, and no evidence of pleuro-pneumonia was found on post-mortem examination of the remaining 24 bullocks.

The sera from 792 bullocks on 12 other properties were then submitted to the test, and four positive reactions were obtained. At the subsequent post-mortem examinations, two of these reactors showed no obvious lesions of pleuro-pneumonia, although, in both cases, patches of thickened pleurae were found, indicating recovery from a previous attack of pleurisy, which we considered had been due to original infection by the pleuro-pneumonia organism. Blood samples taken at the time of slaughter gave negative reactions to the test. It should be noted, however, that these animals were slaughtered six or seven weeks after the original blood samples were taken.

In the other two cases, one animal showed an encapsulated caseating lesion of pleuro-pneumonia in the left lung with pleuritic adhesions between the lung and thoracic wall—a blood sample taken at the time

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of slaughter gave a positive reaction to the test. The other animal showed a healing lesion of pleuro-pneumonia in the left lung, with fibrosis, and an adhesion between the lung and chest wall—its blood sample, while still positive, gave a weaker reaction than that shown in the original test of this animal.

Although 27 months have elapsed since the removal of the reacting bullocks from the herds, no further cases of pleuro-pneumonia have occurred, and the State of Victoria has remained free from this disease.

From our experience with the test of Campbell and Turner, we are confident that it forms a valuable addition to the armamentarium of the live-stock sanitary authorities in the control of bovine contagious pleuro-pneumonia, especially in a country as closely settled as Victoria, where reasonable measures can be taken to limit the spread of the disease.

Two points, however, appear worthy of comment. First, a three-tube test, as suggested by Campbell and Turner, involves a considerable amount of labour, both in the preparation of glassware and in the conduct of the test itself, and in cases where the great majority of animals might be expected to give negative reactions, much of this labour is, we considered, in effect wasted. It was therefore suggested that, in a preliminary test of an infected herd, a two-tube test should be used, sera giving a negative reaction to be discarded, while the sera giving a definite positive reaction should be then run out in a three-tube test. This procedure was actually adopted during the testing of the 817 sera referred to in this report, with satisfactory results.

Secondly, the efficacy of a complement-fixation test is completely dependent upon the suitability of the antigen used. In countries such as the State of Victoria where, in recent years at any rate, pleuro-pneumonia is practically non-existent, but where its introduction from neighbouring States is always a possibility, a supply of suitable natural exudate for the preparation of antigen is impossible to obtain. Moreover, even when prepared according to Campbell and Turner's technique, different antigens vary considerably in their antigenic power. So that test results will be uniform, it is essential that a satisfactorily tested antigen be available to all diagnostic laboratories within the Commonwealth. Until some alternative type of antigen is perfected, it is recommended that the manufacture of antigen for the test be undertaken at a suitable laboratory where a supply of the natural "virus" is readily obtainable, such as the Animal Health Research Station at Townsville.

III. A Cultural Study of the Distribution of the Specific Organism, *Borrelomyces peripneumoniae*,* throughout the Body of Animals Naturally and Artificially Infected.

By A. D. Campbell, B.V.Sc.†

1. Introduction.

For many years, it had been tacitly assumed that the causal organism ("virus") of pleuro-pneumonia was confined to the obvious pathological lesions, i.e., lung, pleuritic exudate, and thoracic lymph glands in natural cases, and the local inflammatory lesions and affected regional lymph glands after subcutaneous inoculation. Dujardin-Beaumetz (1913) had indeed found that, in young calves with specific metastatic synovitis following subcutaneous inoculation, it could be recovered from those lesions also. Reflection might have suggested that the most likely path from the subcutaneous lesion to the joints of the limbs was the blood stream, but its presence was not then suspected in the apparently normal tissues of the body.

However, in 1926, Nakamura, Futamura, and Watanuki demonstrated it not only in the obvious lesions, but also in the circulating blood, liver, spleen, kidney, and lymph glands. Daily sowing of blood on serum agar showed that the organism became septicaemic for considerable periods during the height of the disease.

Soon afterwards, Beller and Tahssin Bey (1926-27) recovered it from the inflammatory pleural, pericardial, and peritoneal fluids of a lamb that died twelve days after birth, the mother having been inoculated subcutaneously 107 days before parturition. From the foetus of another ewe that aborted 180 days after inoculation, it was recovered from the pleural and peritoneal fluids, but not from the heart blood, spleen, and stomach contents. They were also able to recover it from lymph glands, body fluids, spleen, and kidneys of subcutaneously-inoculated cattle. From the urine of a calf, they cultivated it for seven months after subcutaneous inoculation; but not from the liver, heart blood, or bile, when examined *post mortem*.

On the other hand, Yamagiwa, Itabashi, and Ito (1930), in Manchuria, consistently obtained positive blood cultures in four fatal experimental infections for some 10 days before death, but from none of the naturally-infected cases.

Hall and Beaton (1931), by inoculating susceptible cattle with the citrated blood of previously inoculated animals, and by sowing jugular blood into Martin's broth, demonstrated the organism in the circulating

* See Turner, A. W. (1935)—*J. Path. and Bact.* 41 : 1.

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blood from the first day of the thermic response, accompanied by well-marked local reaction, until the local reaction commenced to undergo resolution with a corresponding drop in temperature (4 to 19 days). They were able to cultivate it from spleen, prescapular lymph glands, and nearly every organ of an animal that had died from artificial infection, and from the spleen and lymph glands of all naturally infected animals, but only if the pulmonary lesions were not encapsulated. From naturally infected animals they obtained blood cultures, and claimed not only that the blood stream is invaded until the pulmonary lesion becomes sequestrated, but that the technique of blood culturing is a simple method for detecting and confirming a doubtful clinical examination.

It appeared to us that further and more detailed work was desirable on the distribution of the specific organism throughout the body of infected animals, and on the value of blood-culturing as a diagnostic procedure.

2. Procedure.

To facilitate our studies of bovine pleuro-pneumonia, in particular the complement-fixation test as a means of diagnosis, we had established a naturally-infected herd of cattle by introducing three naturally-infected animals into an uninfected herd maintained in isolation at the Station. Forty-eight days after the introduction, the first case of pleuro-pneumonia in the experimental animals occurred, and was followed by a large number of others. By the introduction of fresh batches of animals to replace infected cases, we maintained the disease in the herd for a period of two years, when the experiment was terminated by the destruction of the remaining animals.

In addition, a number of animals were subjected to experimental inoculation with either pleuritic exudate from natural cases or cultures of the specific organism. We thus had a rich source of material adjacent to the laboratory.

Throughout the investigation, we have made it a practice to conduct, on all autopsied animals, as thorough a bacteriological examination as circumstances would permit. The present communication deals with the cultural examination of the material that became available.

To obtain blood from the living animal for the purpose of inoculation of culture media, an area over a jugular vein was shaved, the animal brought into a draught-free room, the vein distended by compression, the overlying skin painted with iodine, and with a sterile hypodermic syringe 5 ml. of blood were withdrawn. After rejecting 0.5 ml., 1 ml. was introduced into each of four tubes of V.F.-O.S. broth, a special medium described by Turner, Campbell, and Dick (1935).* The tubes were then placed at 37° C., and subsequently examined every 24 hours. Growth is usually obvious in 48 hours, and first manifests itself by numerous pin-point white colonies on the surface of the clot which has formed and is suspended from the glass at the level of the meniscus. Later, these colonies begin to diffuse or stream downwards, and faint, whitish wisps may extend for 3 or 4 mm. On further incubation, the colonies may increase to 1 mm. in diameter, and by

* As this description may be inaccessible to some readers, it is reproduced in the appendix.

extension of the growth into the previously clear medium, a uniform opalescence eventually results. Dark-ground examination of material from such colonies reveals the characteristic mycelial and other elements seen in early cultures of the organism, as described by Turner (1933, 1935). Blood in buffered V.F.-O.S. broth does not give a clot, and consequently no separate colonies form, but a uniform distribution of the organism throughout the medium occurs.

At the post-mortem examination, material was taken from seared organs by means of Pasteur pipettes, and sown into V.F.-O.S., or B.V.F.-O.S. The organism is usually demonstrable within 24 hours in the culture media sown with pleuritic exudate or lung lesions, but from other infected organs and tissues it generally takes several days to appear. The first noticeable growth is often in the form of the so-called comet bodies described by Hall and Beaton (1931), and by Tang, Wei, McWhirter, and Edgar (1935). Microscopic examination of these bodies by dark-ground illumination shows them to be made up of long, tangled, mycelial filaments.

3. Blood Culture.

(i) *Natural Cases (Cattle).*

At the commencement of our studies on the complement-fixation test, the results of which are recorded in the previous paper in this Bulletin, the blood serum of each experimental animal was tested weekly, and, as a check on the results, tubes of media were sown daily with blood from animals within 24 hours of their giving a positive complement-fixation test. As the results did not appear very promising, blood-culturing was discontinued after a time.

Eighty-three blood culture tests were carried out during an investigation on 18 naturally-infected cattle. From 8 animals, 14 positive and 27 negative results were obtained. The analyses of these tests are shown in Table 1. It is obvious that the organism is present in the blood stream most frequently in the early stages of the disease, being found in 6 of the 8 animals examined within 24 hours of their manifesting a positive complement-fixation test. There is, however, little or no correlation with clinical manifestations. That the occurrence of the organism in the blood is intermittent is shown by these results. The irregularity of the results as compared with the experiences of the Japanese workers cannot be ascribed to our medium, which is very sensitive, and frequently gives growth with 10^{-10} or 10^{-12} ml. of a culture.

TABLE 1.—DAILY BLOOD CULTURE TESTS.

Animal Identification No.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	Total No. Tests.	No. Positive.	No. Negative.
2	+	—	—	—	—	—	—	—	—	..	9	1	8
21	—	—	+	—	..	..	..	..	..	..	4	1	3
25	+	—	—	—	+	—	—	—	—	—	10	2	8
31	+	+	—	+	..	..	..	..	..	..	4	3	1
80	+	+	+	+	..	..	..	..	..	..	4	4	0
83	+	—	—	—	..	..	..	..	..	..	4	1	3
91*	—	—	—	—	followed by an interval of 18 days when a + result result was obtained						5	1	4
405	+ only one blood culture test carried out										1	1	0

* Negative series taken before manifestation of clinical symptoms.

Four to six blood-culture tests were carried out on consecutive days on ten other cattle with negative results. These tests were carried out before and during manifestations of marked clinical symptoms.

(ii) *Artificially-infected Animals.*

(*a*) *Cattle.*—Blood cultures were obtained 24 hours after the subcutaneous inoculation of 5 ml. of culture or pleuritic exudate either in front of or behind the scapula. The occurrence of the organism in the blood stream in sufficient numbers to be demonstrated was intermittent, but was more frequent than in natural cases. As an example, we may cite one animal from which the organism was recovered from the blood stream on the second, third, fifth, and seventh days after inoculation, but could not be demonstrated on the fourth, sixth, and eighth days. The disappearance of the organism from the blood stream corresponded with the beginning of resolution of the subcutaneous lesion.

The Willemsian method of vaccination at the tip of the tail is widely practised in North Australia, both during outbreaks of pleuropneumonia and prophylactically before cattle are droved over the long stock routes on their way to markets. It is, therefore, important to know whether, following tail inoculation, the organism may invade the circulatory system. As a normal reaction is confined to a well-defined swelling, extending 2 or 4 inches up from the tip, one would not expect the organism to invade the blood stream.

Four cattle, each approximately 18 months old, were inoculated subcutaneously with 0.2 ml. of virulent culture 1 inch from the tip of the tail. Blood-culture tests, taken daily for 15 days, failed to demonstrate the causal organism. The tail swellings in these animals were very marked from the 5th to the 12th day, after which they commenced to subside. Another animal, inoculated in the tail with pleuritic exudate from a natural case, showed on the 14th day a very oedematous swelling extending into the anal folds. Blood-culture tests were carried out, with negative results, from the 14th to the 20th day. On the 20th day the posterior third of the tail sloughed off. Resolution then rapidly occurred in the upper two-thirds. In the limited number of tail-inoculated animals on which we have carried out blood-culture tests, we have therefore failed to demonstrate the presence of the organism in the blood. Unfortunately, we were only able to carry out cultural tests on one animal in which the reaction was severe, but even in this case the organism could not be demonstrated in the blood stream. It is quite conceivable that, when a very severe reaction occurs, and the gluteal fascia and muscles are invaded, the organism may invade the blood stream.

(*b*) *Sheep and Goats.*—In sheep, positive blood-culture tests were obtained 24 hours after subcutaneous inoculation behind the scapula with either virulent culture or pleuritic exudate. The appearance of the organism in the blood stream was intermittent, but more frequent than in cattle. Its disappearance from the blood stream corresponded with the beginning of resolution of the subcutaneous lesion. The results of the tests are shown in Table 2.

TABLE 2.—BLOOD CULTURE TESTS ON SHEEP FOLLOWING SUBCUTANEOUS INOCULATION.

Animal.	Daily Test Following Inoculation.													Tests on Each Animal.		
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	Total.	No. +	No. —
1	—	+	—	—	+	—	—	—	+	—	+	+	+	13	6	7
2	+	+	—	—	+	+	+	—	—	—	+	+	+	13	8	5
9	+	+	+	+	+	—	—	—	—	—	—	—	..	12	5	7
10	+	+	+	+	—	—	—	—	—	—	—	—	..	12	4	8

Animals 1 and 2 were inoculated behind the scapula with 5 ml. of pleuritic exudate, and animals 9 and 10 with 5 ml. of culture. Blood-culture tests were commenced 24 hours after inoculation. Nos. 1 and 2 were destroyed on the 27th and 22nd days respectively after inoculation, and No. 10 was discharged from the experiment on the 17th day, after complete resolution of the lesion.

Six goats were inoculated subcutaneously behind the shoulder with 5 ml. of culture. Positive blood-culture tests were obtained from each animal 24 hours after inoculation, and subsequently every day until the 8th day, after which the traumatic fibrous reaction surrounding both jugulars prevented further satisfactory tests.

4. Examinations *Post Mortem*.

(i) *Lesions and Organs from Natural Cases.*

Acute, chronic, and recovered cases were examined immediately after slaughter or death.

(*a*) *Acute Cases.*—In Table 3 are recorded the results of a number of examinations of tissues from 23 cattle which had either died of acute contagious pleuro-pneumonia or had been killed in its early stages.

TABLE 3.

Organ or Tissue.	No. of Animals Examined.	Positive Cultural Findings.	Negative Cultural Findings.	Percentage of Positives.
Lung lesions	23	23	..	100
Pleuritic exudate	18	18	..	100
Heart blood	15	9	6	60
Mediastinal and bronchial lymph glands	23	23	..	100
Amniotic fluid	2	2	..	(100)
Gelatinous oedema from epidural space	6	4	2	66.6
Spleen	22	13	9	59
Liver	22	12	10	54.5
Kidneys	22	11	11	50
Cerebro-spinal fluid.. ..	6	3	3	50
Brain	6	3	3	50
Bone marrow (long bones) ..	6	1	5	16.6
Urine	7	..	7	0
Bile	6	..	6	0

In addition to the examinations recorded in the table, filtrates of emulsions of tissue have been used for the inoculation of culture media where there has been danger of bacterial contamination. The organism was thus recovered, after filtration through an L2 candle, from a specific lung lesion of a case that had died overnight, and from a bronchial swab from a case showing acute lesions and gangrenous pneumonia.

It was not recovered from the brain and other organs of a foetus from one of the animals, although it was found in the amniotic fluid.

(*b*) *Chronic Cases.*—Twelve such cattle were killed and examined. Each of eight had a sequestrated pulmonary lesion from which the organism was recovered, but the lymph glands, heart blood, and internal organs were sterile. From another case, which had a large sequestrated lesion, the organism was found in the sequestrum, bronchial, and mediastinal lymph glands, but not in the kidneys, liver, spleen, or heart blood. The remaining three cases each showed a resolving lesion without sequestration, from which the organism was cultivated, though other parts of the body, including the pulmonary lymph glands, were evidently free.

(*c*) *Recovered Cases.*—Thirty-four animals, which from their serological and clinical history had previously suffered from pleuro-pneumonia, but had recovered, were examined *post mortem*. Thirty-three showed either pleural adhesions with fibrosis of a portion of one of the lobes of the lungs, or (in a few cases) only a small area of adhesion. In only one, apparently a rare case of complete resolution, were no lesions found.

Culture tests were made of material from lungs, lymph glands, heart blood, and internal organs, but the organism was not recovered in any case.

(ii) *Artificially-infected Cattle.*

(*a*) *Subcutaneous and Intradermal Inoculation.*—Seventeen cattle were inoculated either subcutaneously or intradermally with culture or pleuritic exudate, and were killed at periods varying from one to several weeks after inoculation. Nine of these animals developed subcutaneous reactions, seven after subcutaneous and two after intradermal inoculation. At autopsy, the organism was recovered in each case from the subcutaneous oedema and from the prescapular lymph gland. In those cases in which the internal organs were examined, it was recovered from the spleen, kidneys, liver, and heart blood, and in one case was also present in the mediastinal and bronchial lymph glands. It was recovered from the synovial fluid of the right carpal joints in three cases. The thoracic cavity of another animal contained approximately 100 ml. of inflammatory exudate, from which the organism was recovered.

(*b*) *Peritracheal Inoculation.*—Two pregnant heifers were inoculated with 10 ml. of pleuritic exudate in the peritracheal fascia of the posterior third of the trachea, and a calf similarly with 10 ml. of culture of a three-day third-generation subculture in B.V.F.-O.S. broth.

One of the heifers aborted two foetuses 33 days after inoculation. The organism was recovered from the heart blood, liver, spleen, and brain, but not the kidneys, of each foetus. The mother was killed the same day. Cultures were obtained from the liver and from heart blood, but not from any other internal organ or from any lymph gland.

The other heifer was destroyed 21 days after inoculation. Cultures were obtained from lymph glands, heart blood, liver, spleen, kidneys, pleuritic exudate, and amniotic fluid. They were obtained also from the heart blood and liver, but not from the kidneys or spleen of the foetus.

The calf, aged approximately six months, was destroyed 50 days after inoculation. Cultures were obtained from the pleuritic exudate, mediastinal and bronchial lymph glands, but not from the heart blood, liver, spleen, or kidneys.

(*c*) *Intrapulmonary Inoculation.*—A steer was inoculated intrapulmonarily with 10 ml. of pleuritic exudate mixed with 10 ml. of a 5 per cent. solution of $CaCl_2$. Death resulted from the infection 17 days later. Cultures were obtained from the mediastinal and bronchial lymph glands, pleuritic exudate, pericardial fluid, and the synovial fluid from the coxo-femoral joints, but not from the heart blood, liver, spleen, and kidneys.

(*d*) *Intravenous Inoculation.*—Five cattle were inoculated intravenously, either with culture *plus* agar, blood culture or emulsified lung lesion, in an attempt to reproduce the disease with the aid of pulmonary embolism, as suggested by Daubney (1933).

One was destroyed fourteen days after inoculation, and cultures were obtained from the lung lesion, mediastinal and bronchial lymph glands, pleuritic exudate, heart blood, liver, spleen, and kidneys.

Another, slaughtered and autopsied 22 days after inoculation, showed a small resolving lung lesion from which a culture was obtained.

The lungs of a third animal, slaughtered and autopsied 36 days after inoculation, showed two small lung lesions, one encapsulated, the other resolving. Cultures from both these were obtained.

The fourth and fifth, killed 32 and 36 days respectively after inoculation, showed no lesions, nor could the organism be recovered from any of the internal organs.

(iii) *Artificially-infected Sheep, Goats, and Rabbits.*

Three of the sheep used in the experiment mentioned in Table 2 were killed and material collected for cultural examination. Two had been inoculated subcutaneously behind the shoulder with 5 ml. of pleuritic exudate. One was destroyed 22 and the other 27 days later. The organism was recovered from the subcutaneous lesions, heart blood, liver, spleen, and kidneys, but not from the brain, bile, and urine, although between the 2nd and 13th day after inoculation intermittent positive blood cultures had been obtained.

The third sheep, No. 9, inoculated subcutaneously behind the right scapula with culture, was destroyed on the 17th day, and all cultural tests failed to reveal the organism in any part of the body, although positive blood culture tests had been obtained from the second to the fifth day, but thereafter had been negative.

A pregnant goat inoculated subcutaneously in the right axillary fascia with pleuritic exudate was killed four weeks later. A culture was obtained from the amniotic fluid, but not from the heart blood, liver, spleen, and kidneys of either mother or foetus.

Two rabbits were inoculated subcutaneously with natural pleuritic exudate. One (a pregnant doe) was killed three days later. Cultures were obtained from the heart blood, liver, spleen, kidneys, brain, peritoneal, and amniotic fluids, but not from the mammary glands.

The other was killed ten days after inoculation. The organism was not recovered from the blood, liver, spleen, kidney, brain, or peritoneal fluid.

5. Discussion and Conclusions.

The opinion of Hall and Beaton (1931) that blood culture offers a "simple method for detecting and confirming a clinical examination" could not be confirmed by us. As already noted, we attempted to use this method to confirm positive reactions to the complement-fixation test. Eighteen such reactors in our naturally-infected herd were submitted to a total of 83 blood culture tests. During some period of the test, all the animals were showing clinical signs of the specific infection, yet only in 14 of the 83 tests was the organism recovered by means of a highly sensitive medium.

By culture tests on cattle, sheep, and goats inoculated with either natural pleuritic exudate or culture, we were able to demonstrate the organism intermittently in the circulating blood from 24 hours after inoculation until death occurred or resolution of the local lesion commenced.

Cultural tests carried out by us on 23 cattle dead of acute contagious pleuro-pneumonia, or killed in the early stages, showed that the causal organism was always present in affected lung tissue, pleuritic exudate, mediastinal and bronchial lymph glands, frequently in the heart blood, liver, spleen, and kidneys, and on one occasion in a long bone. Furthermore, in four cases, we were able to recover it from brain and cerebrospinal fluid and inflammatory oedema in the epidural space (Turner and Campbell, 1935). Contrary to the finding of Beller and Tahssin-Bey (1926-27), we were unable to demonstrate it in the urine of animals affected with acute C.P.P., or those inoculated subcutaneously with either virulent culture or pleuritic exudate, although we found it in the kidneys in 50 per cent. of the cases.

As was to be expected from the work of Tang, Wei, McWhirter, and Edgar (1935), in which they demonstrated the bile-solubility of the organism, we found the contents of the gall-bladder to be free of infection, although it was present in the liver of over 50 per cent. of the cases.

Beller and Tahssin-Bey (1926-7) reported two cases of infection *in utero* following the inoculation of pregnant ewes. Two naturally-infected heifers were found by us *post mortem* to be pregnant; from both animals we obtained cultures from the amniotic fluid, but not from the internal organs of either foetus.

Two pregnant heifers were inoculated in the posterior peritracheal fascia. One of these aborted twin foetuses, and cultures were obtained from their brains, mediastinal, and bronchial lymph glands, livers, and spleens. At post-mortem examination of the other heifer, cultures were obtained from the amniotic fluid, the heart blood and liver of the foetus.

Amongst smaller experimental animals, the organism was recovered from the amniotic fluid of a goat and of a rabbit killed three days after inoculation.

On serological and clinical history, we diagnosed 12 beasts as chronic or "lunger" cases. On post-mortem examination, 9 were found to have well-encapsulated lung lesions. In 8 of these, the organism was recovered only from the sequestrum, while in the other (with a large encapsulated lesion) it was recovered from the bronchial and mediastinal lymph glands as well as from the sequestrum. The remaining 3 animals showed resolving lung lesions without sequestration, and from these lesions the organism was recovered, but not from the lymph glands or tissue. It is possible that, if slaughter had been delayed for some time, complete resolution would have occurred in these 3 cases. The 9 cases with sequestrated lesions had shown no outward signs of the disease for a period of about 3 to 10 months, and constituted potential sources of infection to healthy animals.

Cultural tests failed to reveal the presence of the organism in the glands and internal organs of 34 cattle that at one period of their history were evidently infected, as determined by complement-fixation tests and clinical evidence. Post-mortem examination of these cases revealed only pleural adhesions or fibrosis of a portion of one of the pulmonary lobes and the organism could not be recovered. Such cattle could not be regarded as potential sources of infection.

Cultural examination of cattle experimentally inoculated by various methods showed that the specific organism could be recovered from the resulting lesions, neighbouring lymph glands, internal organs, inflammatory exudates, and from the carpal joints of those animals that developed specific arthritis.

Following intravenous inoculation of ground-up lung lesions of natural cases, we succeeded in producing specific lesions in the lungs of cattle, and the cultural findings were similar to those obtained from naturally infected cases.

We succeeded in recovering the organism from subcutaneous lesions, heart blood, liver, spleen, and kidneys of inoculated sheep, confirming the findings of Beller and Tahssin-Bey.

6. Acknowledgments.

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APPENDIX.

METHOD OF PREPARATION OF THE V. F. CULTURE MEDIA.

The media are based upon a pig-stomach acid-digest of ox-muscle and ox-liver, subsequently enriched with 10 per cent. of ox-serum.

In the following description, V.F. stands for *viande-foie*, B. for buffered, and O.S. for ox-serum.

(*a*) *Preparation of V.F. Acid Digest.*

The following constituents are taken in the following proportions:—

Lean ox-muscle	100 grammes
Normal ox-liver	100 grammes
Pig's stomach	120 grammes
Concentrated hydrochloric acid C.P.	10 ml.
Tap water	1,000 ml.

The ox-muscle is prepared in the usual way, free from fat and aponeuroses; normal livers are selected and freed from large blood vessels, bile ducts, and other removable extraneous matter; and the pig's stomachs are gently washed under running water and freed from scraps of fat, omentum, &c Each is finely minced, mixed in the required amounts, and placed in 6-litre wide-mouthed bottles provided with rubber stoppers. The water is added, then the strong acid, the whole being stirred meanwhile. The bottles are stoppered and placed in a large water-bath maintained at a constant temperature of 50°C. by means of some form of thermoregulator for 24 hours. At the end of this period, a complete peptic digestion has occurred, and usually only a little sludge remains at the bottom of the jars. In order to inactivate the pepsin, the temperature of the water-bath is thereupon raised to 80°C., and the digest filtered through moistened Chardin filter papers, placed in bottles, and heated again to 80°C. The product, which has a clear, light, yellowish-brown colour, is referred to as V.F. *acid digest. Until needed, it is kept in a refrigerated room, where it* remains usable for many months.

(*b*) *Preparation of B.V.F.-O.S. Broth.*

In broths prepared from certain digests, the pleuro-pneumonia organism produces an excessive increase in the hydrogen-ion concentration, and, as this is inimical to the viability of cultures, it was found desirable to keep the pH up to a safe limit by the incorporation of buffer salts in the medium.

Cold acid digest is filtered to ensure the removal of fat, and is then heated to 80°C.; sufficient of a 10 per cent. solution of sodium hydroxide (usually 40 to 50 ml. per litre) is added to raise the pH to 7.5 or 7.6 when tested at approximately incubator temperature in a Hellige comparator with phenol-red indicator. The mixture is held at 80°C. for 15 minutes to facilitate the aggregation of the "earthy phosphate" precipitate, and then 1 per cent. of a mixture of buffer salts calculated for pH 7.4 is added and dissolved. It is then kept between 18°C. and 19°C. (in the ante-room of a refrigerator chamber) for at least 4 hours, and is best left there overnight, in order to complete the aggregation of the precipitate. It is then filtered through Chardin paper, the pH is recorded (it should not be far removed from 7.4), and one-ninth of its volume of ox-serum is added, thus making a final dilution of 10 per cent. The resulting broth is sterilized by filtration through a Seitz E.K. pad, and is tubed under an inoculating hood with aseptic precautions. It is incubated to test its sterility, and is then known as buffered V.F.-ox serum broth, or, shortly, B.V.F.-O.S. broth.

The buffer salt mixture is prepared by intimately mixing 379.0 grammes of anhydrous disodium phosphate and 90.8 grammes of potassium dihydrogen phosphate, both very finely ground.

In this medium, an excellent growth of the organism is obtained, and whatever the acidogenic propensities of the original digest may be, the pH never falls beneath 7.0. Other sera may be used instead of ox-serum for special purposes (e.g., horse, sheep, goat, &c.), with corresponding alteration in the name of the media (i.e., B.V.F.-H.S., B.V.F.-S.S., B.V.F.-G.S., &c.)

(*c*) *V.F.-O.S. Broth.*

For certain purposes (e.g., fermentation tests), it may be undesirable to have the buffer salts in the medium, and, providing the original digest is not excessively acidogenic, the ordinary unbuffered V.F.-O.S. may then be used.

The procedure is as for B.V.F.-O.S. excepting that the buffer salts are omitted.

In a satisfactory batch, the pH will not fall beneath 7.2 or 7.1, whereas if unsuitable digest is used the pH may fall as far as 5.6. The cause of variation in acidogenicity is not yet fully known, but is being investigated.

Explanation of Figures: The varying heights of the columns indicate the limits to which titrations were carried in each case depending upon convenience or the expectations of titre. Black indicates ++++, hatching to the right +++, horizontal ++, to the left +, and no hatching —. Those columns without an unhatched space at the top represent titrations that were not carried out to completion.

CASE No. 364.

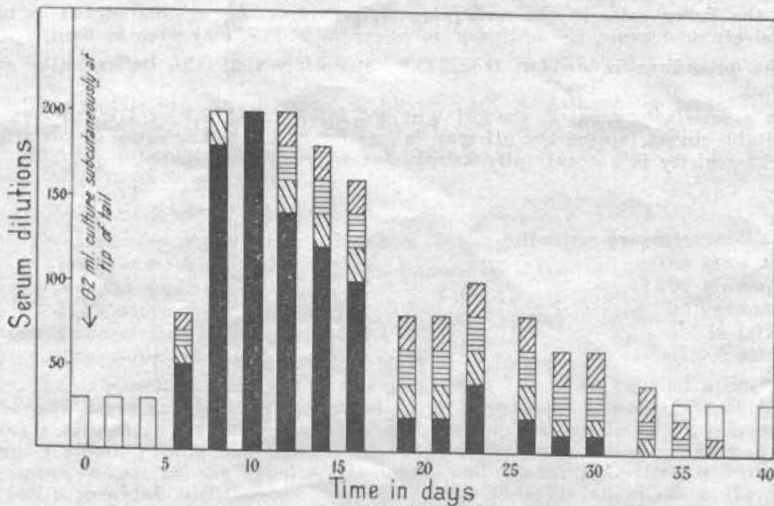

FIG. 1.—Illustrating a typical complement-fixation response following tail inoculation, accompanied by normal local reaction.

CASE No. 21.

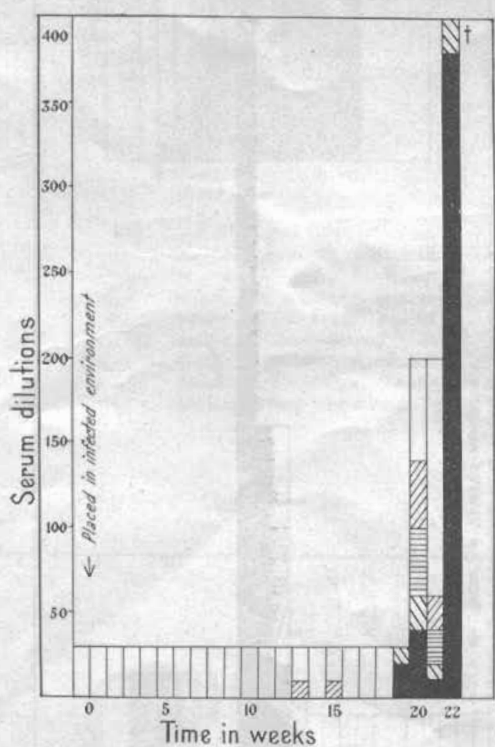

FIG. 2.—Unvaccinated. The figure illustrates the high degree of complement-fixation in a fatal acute case commencing nineteen weeks after entering the infected environment.

Case No. 83.

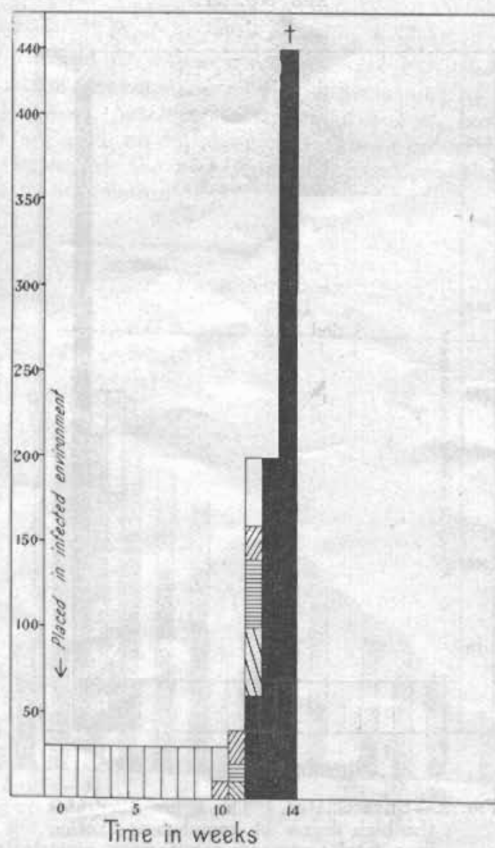

Fig. 3.—Unvaccinated. Illustrates the high degree of fixation in a fatal acute case commencing eleven weeks after entering the infected environment.

CASE No. 168.

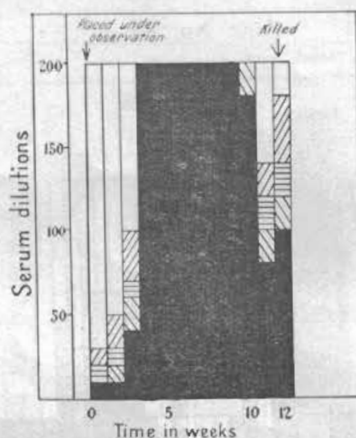

FIG. 4.—Calf aged six months. Natural case in outbreak in dairy herd. When killed *in extremis* twelve weeks after being placed under observation, it showed an encapsulated pulmonary lesion, containing living organisms.

CASE No. 11.

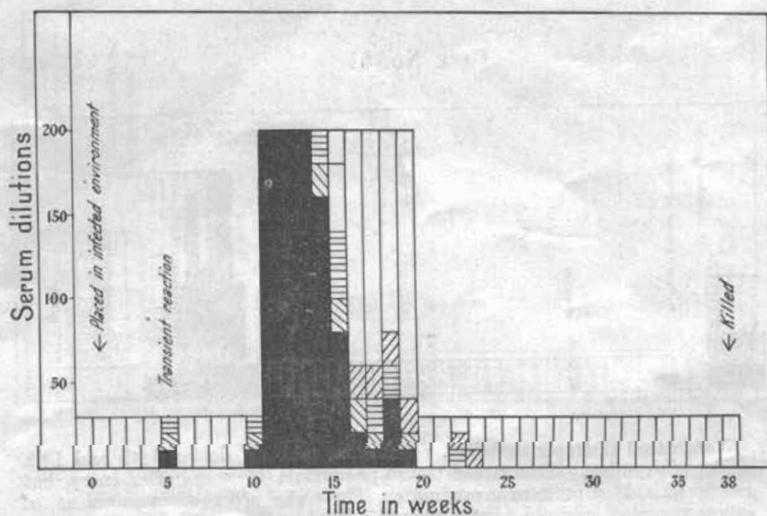

FIG. 5.—Unvaccinated. Symptomless transient reaction five weeks after entering infected environment. Developed clinical pleuro-pneumonia ten weeks after entry, but recovered. When killed 28 weeks after commencement of attack, showed only old fibrous pleural adhesions.

CASE No. 85.

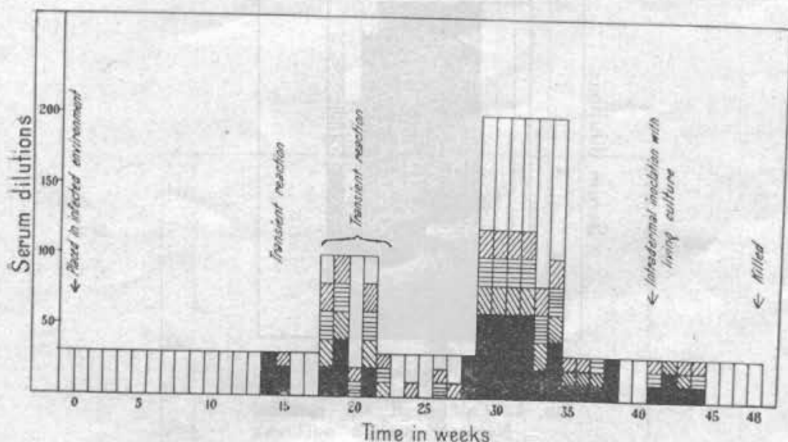

FIG. 6.—Unvaccinated. One symptomless transient reaction during 14th and 15th, and another from the 18th to 22nd week after entering the infected environment. Developed pleuro-pneumonia 28 weeks after entry, but recovered. The intradermal inoculation in this case apparently caused the temporary appearance of a positive reaction. Post-mortem examination 20 weeks after the commencement of the illness revealed only old fibrous pleural adhesions.

CASE No. 61.

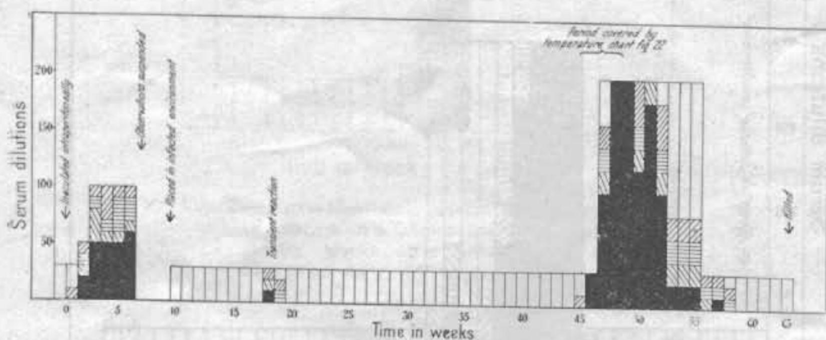

FIG. 7.—Vaccinated (intraperitoneally). Transient reaction during 18th and 19th weeks after entry. Contracted pleuro-pneumonia 46 weeks after entry, but recovered. Post-mortem examination 17 weeks after commencement of illness revealed only old fibrous pleural adhesions.

CASE NO. 2.

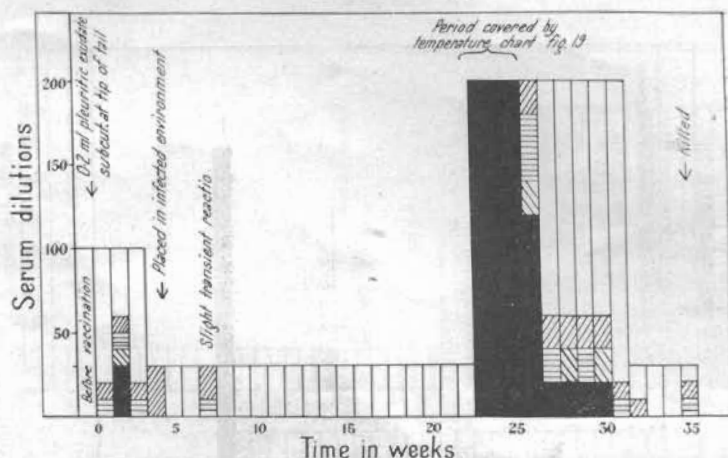

FIG. 8.—Vaccinated (tail). Developed pleuro-pneumonia nineteen weeks after entering the infected environment, but recovered. Examined *post mortem* 13 weeks after commencement of disease it showed only old fibrous pleural adhesions.

CASE NO. 244.

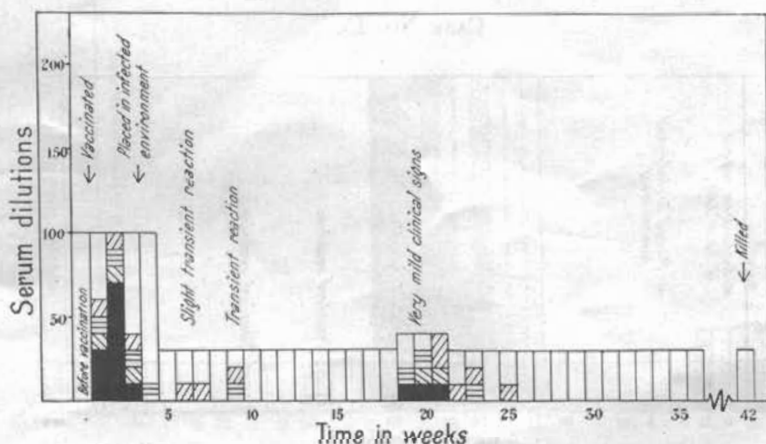

FIG. 9.—Vaccinated (tail). Sixteen weeks after entering infected environment developed very mild pleuro-pneumonia. Showed almost complete resolution (fibrous pleural adhesions) when killed 21 weeks after cessation of symptoms. Note two slight transient reactions soon after exposure.

CASE No. 264.

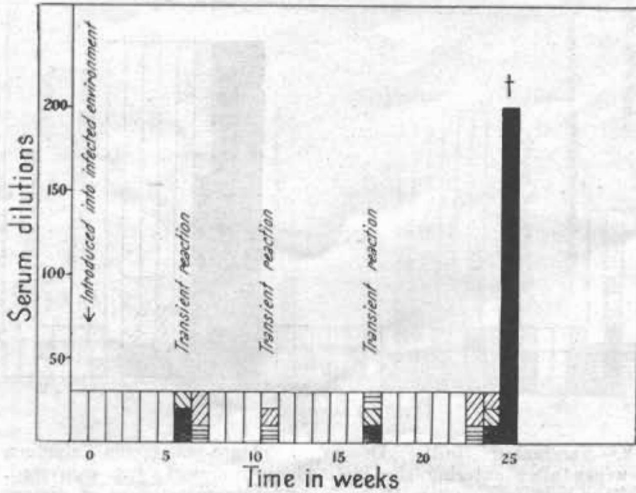

FIG. 10.—Unvaccinated. It gave three symptomless transient complement-fixation reactions before finally developing fatal pleuro-pneumonia 24 weeks after being placed in the infected environment.

CASE No. 15.

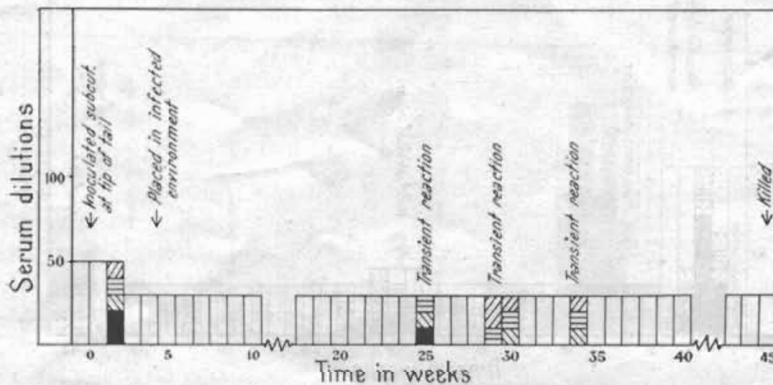

FIG. 11.—Vaccinated (tail). Remained resistant to pleuro-pneumonia, but experienced three symptomless transient c.f. reactions. Post-mortem examination quite negative for pleuro-pneumonia.

CASE No. 89.

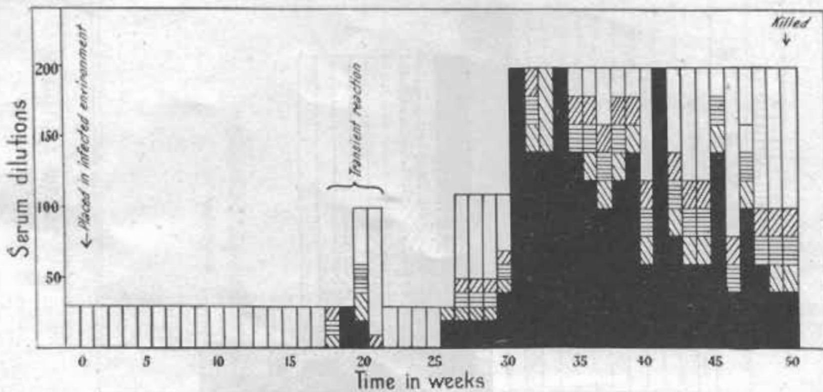

FIG. 12.—Unvaccinated. Symptomless transient reaction between 18th and 26th weeks after introduction. Developed pleuro-pneumonia 26 weeks after introduction, and recovered. Killed 24 weeks after commencement of attack and showed encapsulated pulmonary lesions containing living organisms.

CASE No. 22.

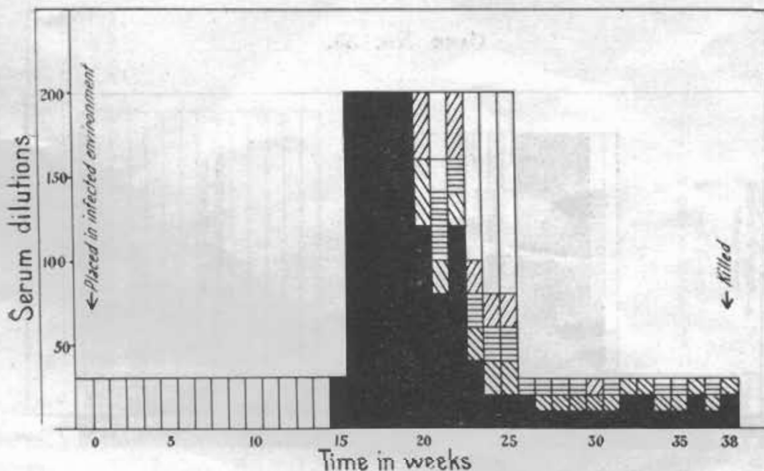

FIG. 13.—Unvaccinated. Developed acute pleuro-pneumonia 15 weeks after introduction. No transient reactions. When killed 23 weeks after contracting the disease a sequestrum containing viable organisms was found in the lungs.

CASE No. 235.

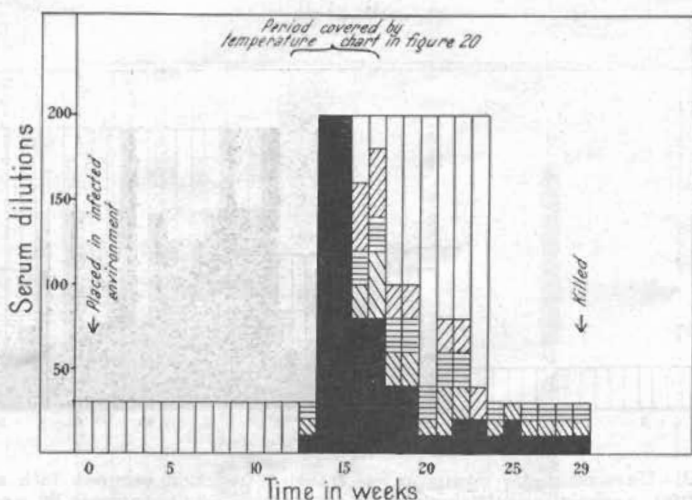

FIG. 14.—Unvaccinated. Thirteen weeks after introduction into the infected environment developed sub-acute pleuro-pneumonia from which it apparently recovered, but when examined *post mortem* had a poorly encapsulated lesion containing viable organisms in the lungs. The temperature chart covering the clinical period is given in Fig. 20.

CASE No. 35.

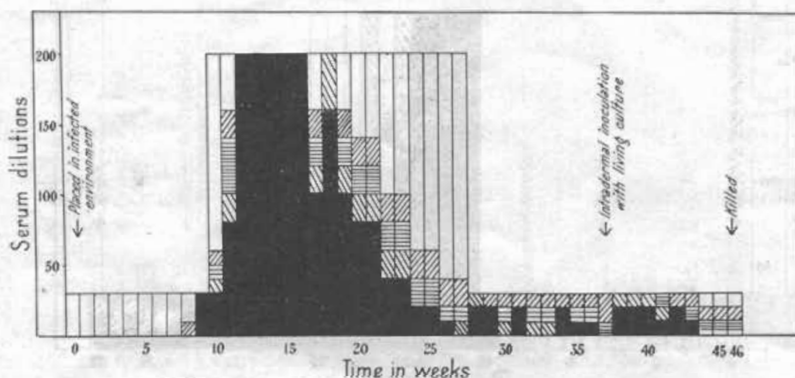

FIG. 15.—Unvaccinated. Acute pleuro-pneumonia 8 weeks after entering infected environment, with recovery. The very small pulmonary sequestrum found *post mortem* was sterile and there were some old fibrous pleural adhesions. There was a brief depression of the c.f. reaction after the intradermal inoculation. Apparently the infection had only recently died out.

CASE No. 193.

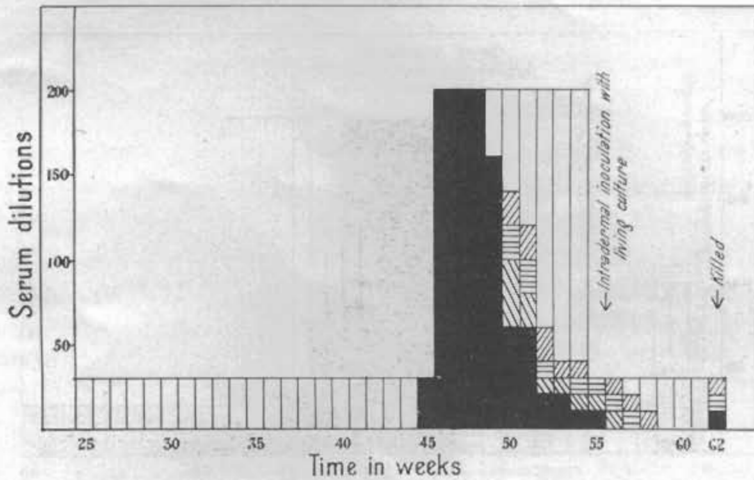

FIG. 16.—Unvaccinated. Forty-five weeks after entering the infected environment, and without having exhibited any transient reactions, it developed a mild attack of pleuro-pneumonia from which it recovered. Ten weeks after the commencement of the disease and when it was still a positive reactor, it was given an intradermal inoculation with living culture; the c.f. reaction gradually declined to negative and did not reappear until seven weeks after the inoculation, when post-mortem examination revealed a small encapsulated lesion containing viable organisms in the lungs.

CASE No. 308.

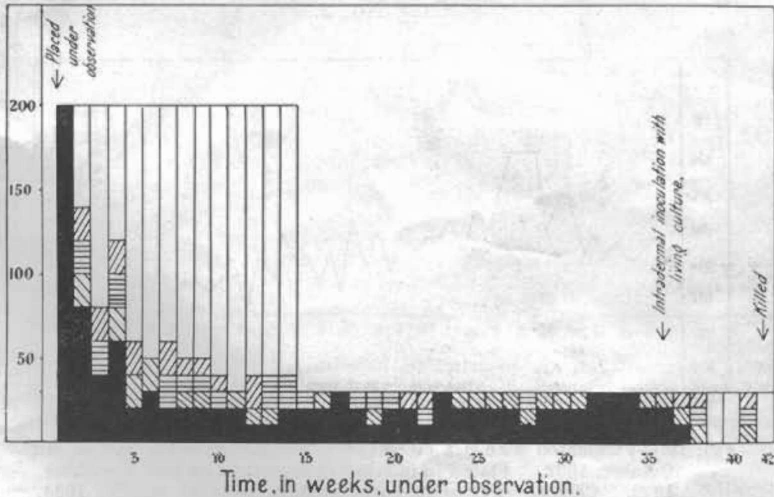

FIG. 17.—Acute pleuro-pneumonia from natural outbreak in dairy herd. The previously persistent positive c.f. reaction was abolished apparently as a result of the intradermal inoculation of living organisms. Post-mortem examination revealed a sequestrum containing viable organisms in the lungs.

CASE No. 31.

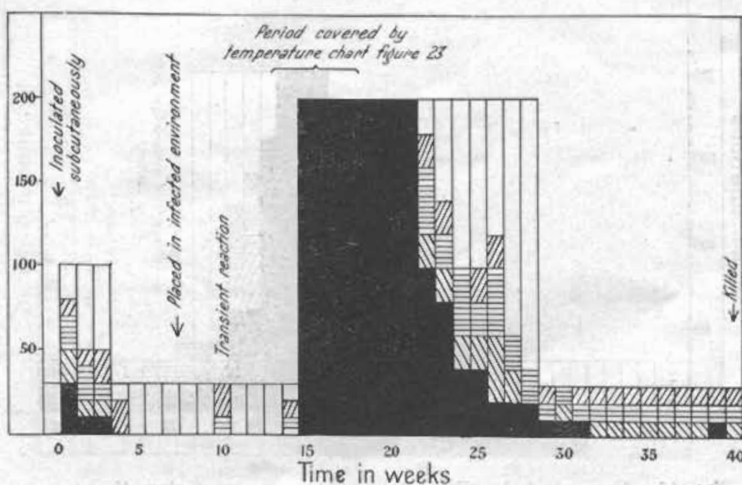

FIG. 18.—Vaccinated subcutaneously behind shoulder. Slight transient reaction three weeks after entry. Contracted acute pleuro-pneumonia (see Fig. 23) eight weeks after entry, but apparently recovered. Post-mortem examination 25 weeks after the commencement of the illness revealed an encapsulated lesion containing viable organisms in the lungs.

CASE No. 2.

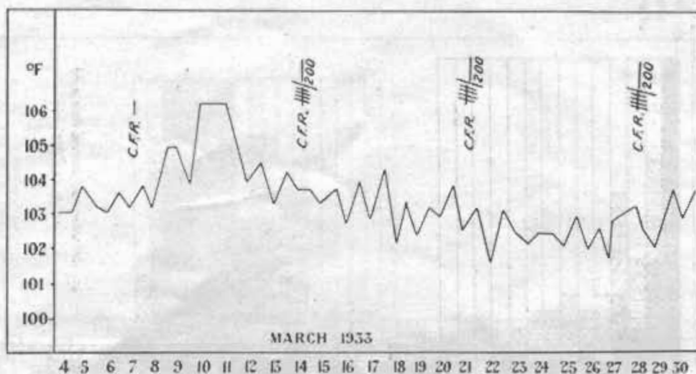

FIG. 19.—Vaccinated with 0.2 ml. pleuritic exudate at tip of tail on 3rd October, 1932. Placed in infected environment on 2nd November, 1932. Clinical and complement-fixation reaction March, 1933. Post-mortem examination revealed old fibrous pleural adhesions. Note the mild temperature reaction. See Fig. 8 for serological history.

CASE No. 235.

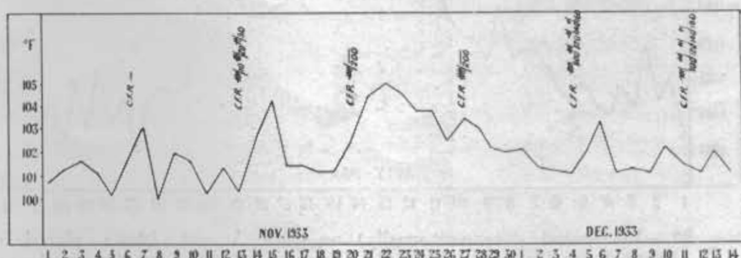

FIG. 20.—Unvaccinated. Placed in infected environment on 16th August, 1933. Developed mild pleuro-pneumonia on 13th November, 1933, and recovered. Post-mortem examination on 14th March, 1934, revealed a resolving pulmonary lesion (compare Fig. 14).

CASE No. 91.

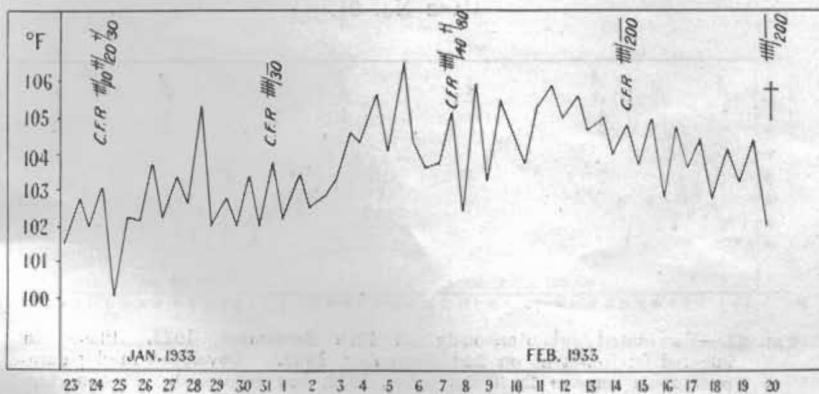

FIG. 21.—Unvaccinated. Entered infected environment on 2nd November, 1932. Developed acute fatal pleuro-pneumonia late in January, 1933, died 20th February, 1933.

CASE No. 61.

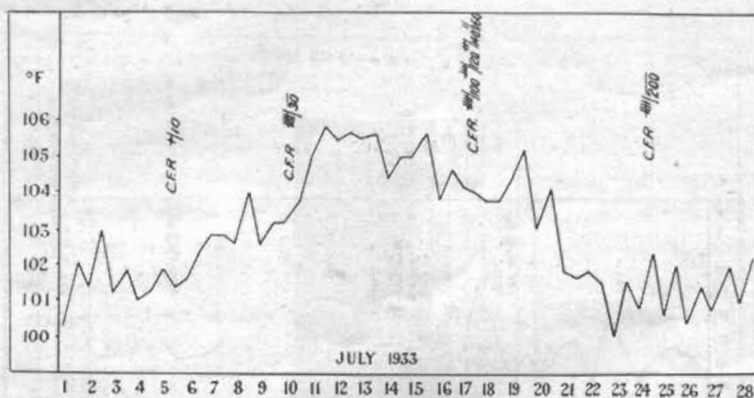

FIG. 22.—Vaccinated (intraperitoneally) on 26th August, 1932; placed in infected environment on 2nd November, 1932; developed pleuro-pneumonia July, 1933, and recovered; killed on 6th November, 1933, when old fibrous pleural adhesions found. Illustrates relationship between clinical and complement-fixation response to acute pleuro-pneumonia (compare Fig. 7).

CASE No. 31.

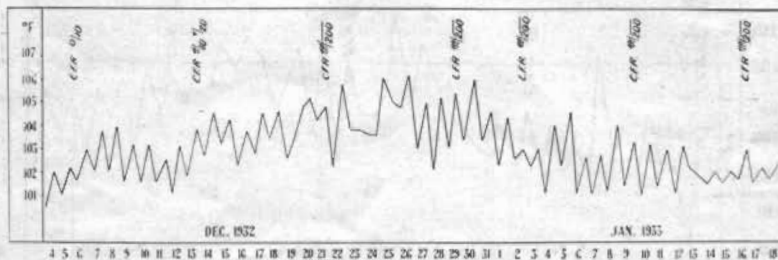

FIG. 23.—Vaccinated subcutaneously on 12th September, 1932. Placed in infected environment on 2nd November, 1932. Developed mild pleuro-pneumonia on the 20th December, 1932, but recovered. Post-mortem examination on 6th June, 1933, revealed small pulmonary sequestrum containing living organisms.

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Govt. Printer, Melbourne.

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