COMPARATIVE STUDIES ON THE SEROLOGY OF SYPHILIS

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PREFACE

The spectacular success of penicillin and other antibiotics in the treatment of syphilis is a frequently quoted achievement of modern public health practice but the efficacy of syphilis control programmes continues to depend upon the performance and results of serologic tests with the patient's serum. The introduction of the Treponema pallidum immobilisation (TPI) test in 1949 solved the problem of specificity to a great extent but the technical complexities of this test led to searches for simpler serologic procedures using other treponemal antigens. Before any of these newer tests can be recommended in routine diagnostic procedures a critical and comparative evaluation of their sensitivity and specificity is necessary.

Although the incidence of infectious diseases like tuberculosis and leprosy has been diminished to a great extent in this country, the number of biologically false positive reactors seems to be increasing gradually, associated, perhaps, with an apparently increasing incidence of the "connective tissue diseases" and tissue sensitivities to the many new drugs now being used. The fall in the incidence of syphilis has also increased the relative numbers and importance of biologically

false positive reactors. A simpler specific serologic test for syphilis will help to identify such reactors in a much easier way so that the false positive reaction could act as an indicator of possible significant systemic diseases which might otherwise go unrecognised.

The work reported in this thesis has entailed detailed studies of sensitivity and specificity of the most promising serologic tests using treponemal antigens prepared from Nichols and Reiter's strain of Treponema pallidum. Serologic reactivities of the different globulin fractions from the sera of syphilitic patients, from experimentally infected rabbits and from biologically false positive reactors were studied extensively in order to find out, if possible, the fractions responsible for the specific and non-specific reactions.

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Chapter I

SURVEY OF LITERATURE
INTRODUCTION

Despite the medical, technical, social and economic advances of the past century, syphilis, yaws, pinta and bejel represent a group of infections which continue to afflict a large proportion of the world population. These infections constitute a vast public health problem and important economic losses in terms of manpower result from invalidism and reduced working capacity, particularly in industrial countries from syphilis, and in agricultural tropical countries from endemic treponematosis like yaws. Amongst these various diseases included in treponematosis, syphilis occupies a major part and dates back to the year 1530 A. D., as evidenced from the poem of Fracastorius, where Syphilus, the name of the shepherd, was infected with the disease. The serology of syphilis, however, has developed only in recent times (1906) but now plays a major role in effective control of the disease from its preventive and curative aspects. Before the introduction of serologic tests, physicians had to depend for their diagnosis solely upon clinical and epidemiological findings and it is easy to imagine how difficult it was to diagnose a case correctly without the help of serologic tests as the disease may simulate the clinical manifestations of other diseases or be

totally lacking in these even when infection is established.

During the past 56 years, serology has developed from the days of Wassermann, with the use of crude lipoid antigen derived from various syphilitic organs of monkey, through the stages of alcoholic extracts of normal heart-tissue and synthetic cardiolipin antigen towards the introduction of treponemal antigen. This continued development of syphilitic serology is mainly due to lack of a specific test with a specific antigen like treponemal antigen. The whole period in the evolution of present-day serology could be conveniently classified into three eras:

1. Period of crude lipoid antigen (1906-1941)
2. Period of synthetic cardiolipin antigen (1941-1949)
3. Period of Treponemal antigen (since 1949)

1. PERIOD OF CRUDE LIPOID ANTIGEN

(1906-1941)

The Wassermann test (Wassermann et al., 1906) for the serodiagnosis of syphilis, was introduced in clinical practice in 1906. The antigen used for the detection of antibodies in the sera of syphilitic monkeys by the complement fixation test, was the saline extract of various syphil-

itic organs of a monkey. A year later Marie and Levaditi (1907) showed that similar complement fixation tests could be performed even by using a tissue extract from non-syphilitic organs. Similar findings were simultaneously reported by different workers e.g. Landsteiner et al. (1907a). Later in the same year Landsteiner and his co-workers (Landsteiner, et al. 1907b) successfully used alcoholic extracts of normal guinea pig heart as an antigen in the Wassermann test. Browning, et al. (1909) recommended the use of lecithin-cholesterol mixtures in Wassermann antigen rather than cholesterolised tissue extract antigen, as they believed that the prospect of more dependable sero-diagnosis in syphilis was bound up with chemically prepared antigens, such as lecithin cholesterol combinations. Sachs (1911) also showed the utility of the addition of cholesterol to increase the sensitivity of alcoholic tissue extracts.

This immunologic reaction, utilising the phenomenon of complement fixation, was modified, refined and simplified through the use of the flocculation technique of Michaelis (1907) who employed a saline extract of syphilitic liver, which was replaced very soon by an alcoholic tissue extract of normal organs (Bruck and Hidka, 1911). Later Sachs

and Georgi (1918) used an alcoholic extract of wet heart muscle plus cholesterol as an antigen.

None of the flocculation tests mentioned above were comparable with the Wassermann test in sensitivity, though it was well known that precipitation tests in immunity could be very sensitive in the detection of minute amounts of antibody. Investigating the reasons for the poor sensitivity of the flocculation test, Kahn (1922a, 1922b, 1922c) concluded that some special laws governed this reaction in syphilis due to the use of tissue extract antigen instead of spirochaetal antigen. After a prolonged study over five years Kahn (1925) found four major factors which favoured precipitation in a mixture of syphilitic serum and antigen. They were: (a) proper concentration of reagents; (b) physical state of the antigen suspension; (c) quantitative relation between the antigen suspension and serum; (d) agitation of serum antigen mixture.

These factors formed the foundation for the development of a practical precipitation test for syphilis, now known as the "Kahn standard test".

Many improvements in the original techniques of complement fixation and flocculation tests were made by subsequent workers, resulting in the introduction of a large

number of newer tests, usually known by the names of their originators like Harrison-Wyler (1929), Kolmer (1922), Price (1948, 1950), Eagle (1937), Hinton (1932), Meinicke (1930), Chediak (1932).

Although it is customary for each author to claim that his test has some special virtue that sets it apart from others, it is nonetheless true that all of these tests depend on basically identical physicochemical and immunologic phenomena. They differ from each other only in minor technical details. It is also true that in a very real and important sense all of these tests are non-specific.

II. PERIOD OF SYNTHETIC CARDIOLIPIN ANTIGEN (1941-49)

The studies of Pangborn (1941, 1942, 1944, 1945a, 1945b) opened the way in the field of purification of antigenic lipids as it was thought that the non-specificity of the reaction in syphilitic serology was due to a defect in the lipid extract antigen, which contains numerous antigenic lipid fractions serologically distinct from one another. These different antigenic fractions are apparently capable of reacting with homologous antibodies present in sera from different sources. For example, one lipid fraction reacts

with homologous antibody in syphilitic serum, another fraction reacts with homologous antibody in horse serum.

Cardiolipin, a non-nitrogenous phospholipid, was isolated from beef heart by Pangborn (1941). Since that time she has perfected its preparation and also described a method of purification of lecithin (Pangborn, 1942, 1944, 1945a, 1945b). The cardiolipin is employed with purified lecithin and cholesterol in specified combinations determined by special titration methods.

Various workers like Faure (1949), Rein et al. (1951), Stevenson (1950), Uroma and Louhivouri (1951) and Vogelsang (1952) isolated sitolipin from wheat germ and used it in antigen preparations in place of cardiolipin.

Baer and co-workers (1950, 1953) introduced synthetic 1-a-dimyristoyl lecithin in place of natural lecithin; the use of this synthetic antigen with known composition helped to standardise and to control the serologic test. The synthetic antigen prepared by Pangborn was used by different workers in different methods of complement fixation and flocculation tests in syphilis. Thus Harris and Portnoy (1944) reported on the use of cardiolipin antigen in the Kolmer Wassermann test while Maltaner and Maltaner (1945)

described the use of cardiolipin antigen in the New York State Health Department complement fixation test. Kahn (1948a, 1948b) after a prolonged trial (1946-48) utilised the antigen in his flocculation test (Kahn standard test).

Microslide tests with cardiolipin antigen were investigated and reported by Rein and Bossak (1946), Kline (1946) and by Harris et al. (1946). The last test is generally referred to as the Venereal Disease Research Laboratory (VDRL) test.

Later a VDRL tube test was reported by Harris et al. (1948) and is now widely used. Hinton, (1949) also reported the use of cardiolipin antigen in his test. Despite these refinements, the tests carried out with synthetic lipoid antigen in the National Serological Evaluation Survey (1949) sponsored by the United States Public Health Service, showed no better results than those using crude lipoid extract, particularly in cases of non-specific or false positive reactions.

Syphilitic Reagin:

The antibody detected in syphilitic serum by these lipoid antigens (crude and synthetic) is called reagin (because it reacts). Opinions differ regarding its immunochemical

constitution, except that it has a molecular weight similar to that of other antibodies. Merklen and Berthaux (1952), Wolf (1957) and Gallego (1959) were of the opinion that reagin was confined mostly within the gamma fraction of globulin, while Moore and Mohr (1952) and Laurell and Hederstedt (1958) observed the association of reagin with the gamma and $beta_2$ fractions. Ottolenghi (1958) clearly explained the position by mentioning the appearance of antibody in the gamma globulin fraction in recent human syphilis which shifts to the beta globulin in the secondary stage and to the alpha globulin in the tertiary stage. This reagin appears in considerable quantity in the sera of animals and man infected with treponemes. It (or a similar substance) is also present in considerable amounts in the sera of many animal species, such as chickens, cattle and horses (Kemp, et al., 1940) and in minute quantity in the sera of all normal human beings (Kahn, 1951). In most normal sera the amount of reagin present is so small as not to be detectable by the standard serologic tests, as these are usually adjusted for sensitivity and specificity.

Unfortunately reagin (or reagin like substance) may increase in amount in the sera of non-syphilitic persons, in

varying incidence, during and after a number of infections with no etiological relationship to syphilis. Thus in a few healthy normal persons (Eagle, 1949) and in many others under the influence of infections other than syphilis, enough reagin may be present in serum to produce positive tests for syphilis with lipoidal antigen in the standard techniques. The tests in these cases are spoken of as biologically false positive tests.

Biologically false positive reactions

The term "biologically false positive reactions" (BFP) has been defined as the reactivity with lipoidal antigens and cardiolipin antigens of sera from patients who do not have syphilis or other treponematosis (Wld. Hlth. Org., 1953). A recently suggested name for such reactive sera in serologic tests for syphilis is reactive "non-treponematic sera". (Wld. Hlth. Org., 1954).

The increased attention to the subject of biologically false positive reactions in recent years, is due to the relative increase in the frequency of such reactions, decline in the number of cases of syphilis as a result of effective control by the treponemicidal antibiotics and the recent development and improvements in the means of differentia-

ting true from biologically false positive reactions by the specific serologic test (Treponema pallidum immobilisation test).

Most of the individuals showing such reactivity in their sera are usually found to have systemic diseases after thorough clinical and laboratory investigations. The acute biologically false positive reactions are attributed to a variety of systemic infections, bacterial, viral, rickettsial or protozoal. They appear during or subsequent to such diseases and regress spontaneously to normality within a relatively short period of time, not exceeding six months. Chronic biologically false positive reactions usually persist for a period of years or perhaps a lifetime. They are not usually due to such precipitating causes (except a few like lepromatous leprosy and chronic pulmonary tuberculosis) as are seen in the acute biologically false positive reactions but reflect an indication of possible significant systemic disease like connective tissue diseases which would otherwise go unrecognised.

No more is known of the actual mechanism causing biologically false positive reactions than is known of the mechanism of a true syphilitic reaction but a reason for this

reaction was suggested by Sequeira (1959) as being due to an antigenic similarity of the lipoidal component of treponemes with the tissue lipoid thus explaining the regular appearance in diseases known to be associated with biologically false positive (BFP) reactions. However, in recent years the occurrence of reagin in serum from cases of connective tissue disease, has been explained by Raffel (1961) as one of the manifestations of the wide variety of antibodies produced in such cases following the establishment of "forbidden clones" associated with genetic mutation of cells in the reticulo endothelial system or alternatively the mutated clones respond more favourably to the autogenous tissue lipoid in the production of reagin in such cases in contrast to the normal person. In a report culled from their vast experience, Moore and Mohr (1952) compiled the following list of non-syphilitic diseases capable of producing biologically false positive reactions.

Infections

(a) Bacterial

Leprosy (Montestrung, 1951; Portnoy, 1952; Beck, 1957 and Mezzadra, 1958)

Tuberculosis, (advanced stage) (Portella, 1953)

Subacute bacterial endocarditis (Josiporis, 1954)

Pneumonia, pneumococcal

Chancroid

Scarlatina

(b) Spirochaetal

Leptospirosis (Montestrung, 1951)

(c) Protozoal

Malaria (Streitmann, 1951)

Trypanosomiasis

(d) Rickettsial

Typhus

(e) Viral - vaccinia (Pianese, 1955; Lunch, 1960)

A typical pneumonia - ornithosis (Loffler, 1959)

Infectious monoculeosis

Lymphogranuloma venereum

Non-infectious diseases or conditions

(a) Collagen diseases (Miller et al., 1954; Moore and Lutz, 1955; Rein and Kelcec, 1957; Eng, 1959)

(i) Lupus erythematosus

(ii) Rheumatoid arthritis

(b) Pregnancy (Wilkinson and Sequeira, 1955; Hartl, 1958; Eng. 1959)

(c) Repeated blood loss

Various special laboratory procedures were devised in the past in the hope of being able to differentiate syphilis from biologically false positive reactions and from other treponemal diseases. Among these were the Hecht verification test (Hecht, 1914), the Wassermann confirmation test (Wassermann, 1921, 1922), and the Whitebsky confirmation test (Whitebsky, 1933, 1938). None of these methods has been able to distinguish consistently between true positive and false positive serologic reactions.

Kahn (1940, 1941, 1943) applied a differential temperature technique in his test for verification purposes and concluded that in a typical false positive type of reaction, precipitation was more marked at 1°C than at 37°C or the precipitates were dispersed at 37°C, whereas reaction patterns were reversed in a typical syphilitic type (Kahn verification test). The author (Kahn, 1950) also agreed that the procedure is not safe enough for unrestricted use in sero-diagnosis of syphilis, although after its proper application it can help in the detection of relatively large numbers

of false positive sera.

The same problem of "non-specific" or "biologically false positive" reactions was also dealt with in the euglobulin-inhibition test of Neurath and his associates (1947). In this test the euglobulin fraction is isolated by isoelectric precipitation under carefully controlled conditions from the test serum. Another serum protein fraction (a by-product of the plasma fractionation programme of the American Red Cross) known as Fraction IV-I contains an inhibitor, which in most instances, inhibits the precipitation reaction with the euglobulin fraction of sera giving false positive reactions in sero-diagnostic tests. Either no inhibition or very little inhibition is observed in the euglobulin fraction of syphilitic sera.

After a prolonged study of 8 years (1942-50) on 5,000 samples of false positive sera, Rein and his colleagues (1950) commented that the Neurath test was the most accurate verification procedure based on non-specific physico-chemical methods although a lack of reproductivity in the test results was reported by Falcone et al. (1953).

Portnoy and his colleagues (1954) devised a simple procedure for differentiation of syphilitic and non-syphilitic

reactions obtained in the serologic tests for syphilis with leprosy sera. With lipoidal antigen suspension containing choline chloride, sera of syphilitic patients (with or without leprosy) showed an increase in sero-reactivity, whereas non-syphilitic reactive leprosy sera showed a decrease in reactivity. They claimed that this procedure compared favourably with Treponema pallidum immobilisation (TPI) test in leprosy patients with or without clinical evidence of syphilis.

The value of these verification methods in distinguishing between a syphilitic and a non-syphilitic serologic reaction is, however, questionable and it has been accepted that there is no 'specific' test for syphilis (or other treponemal disease), with the possible exception of the TPI test employing specific treponemal antigens prepared from Nichols pathogenic treponema pallidum strain.

III. PERIOD OF TREPONEMAL ANTIGEN

(since 1949)

Of the several serologic methods used for verification purposes, the most promising proved to be the Treponema pallidum immobilisation test (TPI) described by Nelson and Mayer (1949). These observers found that sera from persons with syphilis would immobilise the T. pallidum in the

presence of complement. The development of the TPI test followed mainly Turner's (1939) demonstration of protective antibodies in the serum of syphilitic rabbits. Later on, similar antibodies were demonstrated in human syphilitic sera, especially those from persons with secondary or tertiary syphilis (Turner, 1948). But difficulty arose regarding utilisation of the test in routine serology as cultivation of T. pallidum was not possible at that time. Various cultivable non-pathogenic spirochetes had been used in agglutination and complement fixation tests with sera from syphilitic patients which gave non-specific reactions in a large number of cases. Touraine (1912) also tried to utilise T. pallidum from human syphilitic lesions in agglutination reactions. The development of the extraction of virulent T. pallidum from rabbit testicular syphilomata and the maintenance of these organisms in vitro by Nelson and Mayer (1949) led to the possibility of detecting antibody in sera from syphilitic animals and human beings. They used Nichols strain of T. pallidum, which had been isolated from the cerebrospinal fluid of a patient with relapsing neurosyphilis by Nichols and Hough (1913). In spite of having been maintained in animals for about 50 years, it is still able to cause clinical syphilis in man.

The nature of the protective or immobilising antibody (immobilisin) was studied by Turner and others (1948, 1950) although the protective effect of this antibody was contradicted later on by McLeod and Magnuson (1953) as they found experimentally that a new infection may occur in the presence of a positive TPI test. Turner and Hollander (1957) were of the opinion that in respect of the immobilising antibody there was a positive correlation between the amount of this antibody and immunity in experimental syphilis in animals.

Nelson and Mayer commented that the immobilising and reagin activities of a serum were due to the presence of two separate antibodies as reagin could be absorbed from the serum with lipoidal antigen without lowering the titre of the immobilising antibody. Furthermore, since the level of reagin was relatively low in late syphilis when resistance to reinfection was high, it apparently played no role in immunity. On the other hand immobilising antibody appeared to kill T. pallidum and therefore its appearance might be associated with, and possibly responsible for, the development of an immune state. The immobilising antibody was also demonstrated by others (Gastinel and Borel, 1952;

McLeod and Magnuson, 1952, 1953) in sera from animals (rabbits and mice) immunised with live or dead pathogenic treponemes - Nichols strain for instance, but not in sera from animals immunised with live or dead non-pathogenic treponemes such as Reiter's strain (Gelperin, 1951).

Opinions of other workers regarding the immobilising antibody are as follows:

D'Alessandro (1946) demonstrated three different antibodies in syphilitic sera, viz. one antilipoidal and two antitreponemal. Of the antitreponemal antibodies one was thermolabile while the other was thermostable. Durel et al. (1952) reported that in untreated patients with fresh infections the TPI test did not give as dependable results as the "old" sero reactions. They also found that the antilipoidal antibodies, demonstrated with antigens produced from alcoholic tissue extracts (or with cardiolipin alone) appear earlier than treponemal immobilising antibody. McLeod and Magnuson (1953) reported that the TPI test remained positive but the Serologic Tests for Syphilis became negative after treatment for syphilis. Chacko (1953) confirmed the above findings in experimental rabbit syphilis and showed that treponema immobilising antibody was distinct from Wasser-

mann antibody which could be absorbed from syphilitic serum with lipoidal tissue extract antigen leaving immobilising antitreponemal antibody intact.

Hardy and Nell (1955) showed that T. pallidum could be agglutinated by two antibodies, one of which could be absorbed by lipid antigens, presumably the Wassermann reagin, the other being a specific antitreponemal antibody. Thus they concluded that T. pallidum contained not only a lipid antigen related to substances occurring widely in nature which formed the basis of the Serologic . Tests for Syphilis, but also one or more specific antigens. The sharing of two dissimilar antigens with unrelated species would appear extremely unlikely thus explaining the basis of the specificity of the TPI test. Laurell and Hederstedt (1958) studied the immuno-chemical behaviour of immobilising antibody by paper electrophoresis; the migration of the antibody along with the gamma fraction was proved by carrying out the TPI test with the eluted protein fraction.

Nelson and Mayer (1949) commented that as an in vitro technique for the detection and measurement of specific antibody produced during syphilitic infection, the immobilisation test offered a convenient approach to the study of fundamental problems in the biology of diseases in animals

and men, the most important of which was the possible absence of immobilising antibody in non-syphilitic individuals whose sera contained reagin, i.e. the "biologically false positive reactors". These authors carried out the TPI test on a significant number of samples of sera from different stages of syphilis and from non-syphilitic patients with other diseases showing biologically false positive reactions. They concluded that since reagin and immobilising activities appeared to be due to separate antibodies, it was possible that the stimuli which incited the production of reagin in a non-syphilitic individual might not give rise to immobilising antibody, whereas the titre of immobilising antibody unlike reagin would remain relatively high in untreated syphilis, where infection progressed to a latent phase. The immobilisation test was sufficiently sensitive to detect antibody in a fair number of sera from cases of primary syphilis in which comparatively low levels would be expected to be present. Thus the persistent absence of immobilising antibody would help to exclude the diagnosis of syphilis.

From the very beginning the technical difficulties of the TPI test were pointed out in nearly all reports and publications. The most important of these difficulties was due to a lack of standardisation of the "biological" antigen

suspension as it was more variable than antigen suspension prepared from a well-defined stock antigen, such as cardiolipin antigen, that could be kept for years. By repeated testing of the same suspension of treponemes using the same batch of complement, Thiviolet and co-workers (1954) observed the reproducibility of the qualitative TPI test to be much the same as that observed with the usual serologic tests for syphilis. By repeated quantitative testing of one serum, each with different suspensions using different batches of complement, Nelson and Diesendruck (1951), Chorpenning (1953) and Wilkinson (1954) obtained the standard deviations of 25%, 35%, and 20% respectively. To overcome this difficulty some authors (Magnuson and Thompson (1949), Nelson et al. (1953), Ajello et al. (1953), Wilkinson (1954) modified the original technique by utilisation of different dosages of complement and also altering the concentration of different reagents in the survival medium for the preservation of T. pallidum. Regarding the reproducibility of the TPI test Harris (1956) commented that the reactivity level of the TPI test may be varied by: (a) altering the complement content of the test; (b) increasing the amount of thioglycollate in the survival medium; (c) failure to maintain a constant number of organisms in the treponeme suspension used as a test antigen;

(d) use of cortisone to enhance the growth of treponemes in vivo.

These findings were also supported by Sequeira and Wilkinson (1955). The reproducibility of the TPI test after modification of its technique was studied by different workers (Harris et al. (1955), Roy et al. (1953), Sequeira and Wilkinson (1955)), who claimed that the reproducibility of the test from a laboratory aspect and routine use is comparable to that of the STS if certain variable factors like potency of the antigen and titre of the complement used can be standardised.

The clinical value of the TPI test was investigated by different workers (Magnuson and Thompson, 1949; Nelson et al. 1951; Mohr et al., 1950; Durel et al., 1951; Chacko, 1953; Edmudson et al., 1954; Wilkinson, 1954; Sequeira and Wilkinson, 1955; Nielsen and Rein, 1956) including the authors of the test. They were of the opinion that:

1. The TPI test is sensitive enough to detect syphilis in all stages beyond its early primary stage. The test could thus be used as a diagnostic test except in early primary syphilis.
2. The TPI test remains positive indefinitely in syphilis after adequate treatment unless treatment is started very early, whereas the STS usually yielded to treat-

ment. Thus the test was not fit to be used as a test of cure except in treated early syphilis.

3. The TPI test rarely becomes positive in normal persons or in diseases other than syphilis except those due to treponemata. Thus it appeared highly specific and had greatest value as a verification test for the biologically false reactions obtained with standard tests.

Despite the real scientific merit of the TPI test, both its experimental and clinical usefulness had been limited due to the technical complexities which restricted its performance to specialised laboratories. This led to intensive search for other methods of detecting specific antibody to pathogenic treponemes, e.g. the utilisation as antigen of dead virulent T. pallidum from rabbit's testicular inoculum, cultivable avirulent Reiter's treponeme, or an antigenic fraction from either of the two organisms. Thus a large number of modified treponemal tests came into the field within a few years after the publication of Nelson and Mayer's TPI test in 1949. The newer treponemal tests can be conveniently classified into 4 groups according to the nature of the antigenic constituents.

A. Tests with virulent treponemes (rabbit testicle suspension)

1. Treponemapallidum agglutination test (TPA)
2. Treponema pallidum immune adherence test (TPIA)
3. Treponemapa llidum methylene blue test (TPMB)
4. Treponemal complement fixation test (TWR)
5. Fluorescent treponemal antibody test (FTA)

B. Tests with avirulent Reiter's treponeme (artificial culture)

1. Reiter's complement fixation test (RCF)
2. Reiter's fluorescent treponemal antibody test (RFTA)

C. Test with protein fraction of virulent treponeme

Treponema pallidum complement fixation test (TPCF)

D. Test with protein fraction from cultivable Reiter's treponeme

Reiter's protein complement fixation test (RPCF)

A. TESTS WITH VIRULENT TREPONEMES

1. Treponema pallidum agglutination test (TPA)

Tani (1940) showed that T. pallidum (a Japanese strain) under proper conditions would agglutinate in the presence of syphilitic serum. The test was repeated by Cain (1953) using Nichols strain as an antigen; but, unfortunately, the organisms either tended to agglutinate spontaneously or were

agglutinated by sera from persons in whom treponemal infection could be reliably excluded. To obviate the spontaneous agglutination, McLeod and Mugnuson (1953), and Hardy and Nell, (1955) modified the test by using cortisone treated rabbits, special extracting medium, heat inactivated treponemes and a special reagent for dilution of the serum. The reason for a non-specific reaction by the agglutination test was clearly explained by McLeod and Stokes (1955) who demonstrated that the reagin present in rabbit antisera immunised with lipoid reagin aggregates, contains antilipid antibodies capable of aggregating T. pallidum but unable to immobilise it in the presence of complement. Absorption of syphilitic serum with VDRL antigen removed some but by no means all of the agglutinating activities of the serum for pathogenic T. pallidum and without any change of TPI titre. So the experiment suggested that the antibody detected by the TPA test is not similar to that of the TPI test. To eliminate non-specific reactions, Hardy and Nell (1955) proposed the adsorption of reagin by VDRL antigen before proceeding to the TPA test. They found that agglutination results compared favourably with the results of the TPI test in their study on a large number of sera. Opposite views were expressed by Magnuson and

McLeod (1956) who were of the opinion that the agglutination tests do not have a diagnostic value similar to the TPI test as the technical difficulties have not been overcome and interpretation is not clear. In an evaluation study of different treponemal tests Falcone and Harris (1957) found that the TPA test with unabsorbed sera showed a greater number of reactive results than those obtained with absorbed sera, in comparison with the TPI and other treponemal tests. Sequeira (1959) showed that in spite of its simplicity, there are variations in its reproducibility with different antigen batches.

2. Treponema pallidum immune adherence test (TPIA)

A reaction between human erythrocytes and treponemes sensitised by antibody from syphilitic serum, was first described by Nelson (1952a, 1952b). In a later report (Nelson, 1953) he confirmed the specificity of this reaction after testing a large number of sera. The report also stated that the results of the tests correspond with those of TPI tests along with the clinical diagnosis except in a few instances.

Glansky and Harris (1954) after a comparative study of the TPIA, TPI and VDRL tests, came to the conclusion that

the antibody producing reaction in the TPIA test is probably similar to, if not identical with the substance that activates the TPI test, as the pattern of reactivity of the former more closely follows the latter than that of the VDRL slide test. They also suggested that the TPIA test might be a practical substitute technique for detecting immobilising antibody as the test is a simple and rapid laboratory procedure without any requirement of freshly prepared antigen.

By the absorption technique Daguet (1956) showed that the antibody responsible for the TPIA test is different from Wassermann reagin, but is closely allied to immobilising antibody of the TPI test and recommended the application of the test for the detection of specific antibody in syphilitic serum. He also mentioned the suitability of the test because of the very small amounts of killed treponemal suspension required as antigen and for the rapid technique.

In spite of this continuous support for a long period, contradictory reports appeared from different quarters regarding its sensitivity and reproducibility. Falcone and Harris (1957) considered the test as an insensitive one in comparison with the TPI test, while Sequeira (1959) found the test to be lacking in its reproducibility in spite of its technical simplicity.

3. Treponema pallidum methylene blue test (TPMB)

Rosenau and Kent (1956) described the principle of this test, which depends upon affinity of virulent T. pallidum (Nichols strain) for methylene blue dye, as the stained treponemes appear red when observed under darkfield microscopes. Affinity for the stain is lost, however, when treponemes are incubated with syphilitic serum and complement. The results are recorded like the TPI test as the percentage of T. pallidum that appeared red (unsensitised).

In an evaluation study with the different treponemal tests Falcone and Harris (1957) observed that the method of reading the TPI test with methylene blue (TPMB) yielded results which are in agreement with those obtained in the TPI procedure but when the treponemes remain actively motile, this method seems to offer no advantage over the regular technique for reading the TPI test.

4. Treponemal complement fixation test (TWR)

The use of specific treponemes (Nichols strain) as an antigen for the complement fixation test, was studied by different workers (Tuttle and DeBerry, 1955; Magnuson and Portnoy, 1956; Portnoy and Magnuson, 1955; 1956) in the past but its practical application was delayed as most of the workers were afraid of unpredictable results if it is used

without previous treatment with lipid solvent.

In spite of this possibility Price and Whelan (1957) used the heat-killed mechanically disintegrated virulent T. pallidum (Nichols strain) as an antigen for the complement fixation test. They claimed the test as reproducible and found it to be more specific and sensitive than the STS with lipoid antigen. When compared with the TPI test it was found to be similar in sensitivity and specificity. This view was also supported by Price (1958) when he mentioned the advantages of the test as it becomes positive sooner than the TPI in early syphilis and appears to become negative more readily than other tests when the patient is under antibiotic treatment. Unfortunately the same author (Dunlop and Price, 1959) contradicted his own conclusion a year later and commented that due to non-specificity and poor reproducibility of the TWR? it cannot be regarded as a substitute for the TPI test. Laird (1959) was also of the opinion that the TWR is not a substitute for the TPI test in spite of its technical and economic advantage while Wilkinson and Johnston (1959) could not find any agreement between the results of the TWR and TPI tests. Sequeira (1959) considered the test as unsuitable for routine and confirmatory

purposes as it gives an unsatisfactory degree of specificity due to the reaction of an antigen with a fair number of antibodies like reagin, immobilising antibody (immobilisin) and another unidentified factor.

5. Fluorescent treponemal antibody test (FTA)

The credit for the application of a fluorescent test for the detection of treponemal antibody, goes to Harris, Deacon and Smith (1957) who utilised the method of Coons (1942) after conjugation of fluorescent dye with goat antihuman serum and goat antirabbit serum prepared by injection of globulin from the respective sera.

They used the method of Coons and Kaplan (1950) for labelling the antibody with fluorescent dye. The antigen used for the test was killed T. pallidum (Nichols strain) obtained from rabbit testicular tissue by extraction as recommended for the TPI test. The sera used for the test were diluted 1 in 5 in buffered saline after inactivation. The authors studied the sensitivity of the test in human syphilis along with the TPI and TPCF tests and found that the results of the FTA test compared favourably with the TPCF test (complement fixation test with protein antigen from virulent T. pallidum) in sensitivity and was considerably more sensitive

than the TPI procedure. They, however, did not evaluate the specificity of the test amongst non-syphilitic individuals. Harris et al. (1957) suggested that if the specificity was found to be satisfactory after proper evaluation the test could be applied in routine procedure as it yielded no inconclusive or anticomplementary results in comparison with the TPI or TPCF tests. The recommendation was also based on factors like easy availability of antigen, quick testing procedure and preservation of slides at 4°C for future reference.

The indirect fluorescent antibody technique of Weller and Coons (1954) was applied by Borel and Durel (1959) after labelling antihuman globulin serum (Coombs serum) with fluorescein isothiocyanate. The antigen used in the test was similar to that of Harris and others (1957). The authors studied the sensitivity and specificity of the test in a fair number of syphilitic and non-syphilitic subjects in association with the TPI and classic cardiolipin (Kline) tests. In non-syphilitic subjects, the results were identical with those of the TPI test. The FTA test was found to be more sensitive than the TPI in untreated primary syphilis and amongst some treated primary and secondary cases of syphilis. They also found the test to be reproducible.

Deacon et al. (1960) modified the testing procedure by using the sera after dilution at 1:200 (called FTA-200) as they found the test to be sensitive enough to show reactive results in normal sera diluted to 1:100. A weak reactive reading at 1:200 was considered by the authors as insignificant and was described as negative. A comparative study was made by the authors along with other tests like the TPI, RPCF and VDRL slide tests in primary (treated and untreated) and secondary cases of syphilis. In the treated primary group where the FTA titre was low, comparative tests tended to give a variety of results whereas in the untreated group the agreement with the comparative test was found to be satisfactory. In secondary cases they used to get a very high titre in comparison with other tests.

From a comparative study with FTA, VDRL and RPCF tests Montgomery et al. (1960) came to the conclusion that the FTA test became reactive earlier in the course of syphilis than either of the other two tests and followed much the same pattern as reagin with the highest titre in secondary syphilis. They also found a reduction of the FTA titre following treatment though the test remained positive over a more prolonged period than the VDRL.

Wilkinson (1961) thoroughly investigated the sensitivity and specificity of the FTA-200 test in a large number of sera from treated and untreated syphilis (early late and latent) and those from presumed normal individuals and biologically false positive reactors (BFP). In the group of early syphilis, his findings were similar to those of earlier workers (Borel et al., 1959 and Thiovolet et al., 1960). Wilkinson found the FTA-200 test to be as sensitive as other types of tests, such as RPCF and WR in early syphilis and it even showed more sensitive results than the latter tests with the sera from cases showing dark field positive primary lesions. In the early treated group he found the test to remain positive even after the disappearance of reactivity with WR and in latent syphilis the reactivity of the test was found to be too high.

Regarding the specificity of the test he found only 1.5% reactive results in the presumed normal group whereas in the biologically false positive reactors the test showed a close agreement with the TPI test. The latter finding was also supported by Mannucci and Spagnoli (1961).

Though the relationship between the antibodies revealed by the FTA test and the antibodies responsible for immobilisation, agglutination and immune adherence of treponemes, is not yet known, preliminary experiments by Wilkinson (1961) suggested that it is possible to absorb out the reagin

activity of a serum without loss of the FTA activity whereas adsorption with the Reiter's treponemes removes the reactivity of the RPCF test but has no effect on the FTA titre of the serum.

These findings together with negative FTA-200 results with the sera giving non-specific reaction with tissue extract antigens as judged by negative TPI results led him to conclude that the antibody concerned in the FTA test differs both from reagin and from that responsible for the RPCF test. He also mentioned the possibility of interference by reagin in fluorescence of treponemes as the reagin can unite with the same organisms.

B. TESTS WITH A VIRULENT CULTIVABLE REITER'S TREPONEME

1. Reiter's complement fixation test (RCF)

Reiter's treponeme was isolated by Wassermann and Ficker (1922). This organism along with certain other strains was passed on to Reiter for further study. The details of the isolation of this organism were presented by Reiter (1960) who mentioned that the organisms were strongly virulent and were isolated from rabbit's testicles after intravenous injections of infected material obtained from a chancre. Gaetgen (1937) was also convinced that Reiter's

treponeme was a genuine T. pallidum as he mentioned that though the strain was lost in Germany during World War I it was brought back afterwards from the U. S. A. where it was maintained in the meantime.

Whatever its origin may be, it is now distinct from the causative agent of syphilis in that it is avirulent for animals, multiplies in a relatively simple media and survives long periods without subculture.

The use of a phenolised suspension of Reiter's treponeme as an antigen in syphilitic serology, dates back to 1929, when Gaehtgen (1929) introduced it. Despite the conflicting reports about its specificity this test was widely used by continental workers before the Second World War. Puccinelli (1951), D'Alessandro and Dardanoni (1953) extensively investigated the serologic properties of the antigen in the sero diagnosis of syphilis. They found that the protein and polysaccharide fractions of the treponemes react with the specific antitreponemal antibodies in syphilitic serum. The necessity for removal of antilipid antibodies from the sera to be tested, has been avoided by the use of a strain of Reiter organism in which the lipoidal fraction is thought to be antigenically inert (Puccinelli and Pezze, 1949).

Comparison of results given by these tests, using a suspension of intact organisms as antigen, with STS, showed that such suspensions have a higher specificity and sensitivity than lipoidal antigen used in the STS. (Benazat et al. 1954; Sohier et al., 1954; Hardy et al., 1955; Gastinel et al., 1956). Suspension of Reiter's treponemes disintegrated by ultrasonic waves has also been found to be effective (Fuhner and Gaehtgens, 1929; Vaisman et al., 1958).

Wilkinson (1957) introduced the use of mechanically disintegrated Reiter's treponeme suspension as an antigen for the complement fixation test. He also carried out parallel tests on a large number of sera with the TPI and the standard lipoidal antigen and came to the conclusion that the RCF test is more specific and sensitive that the STS as the results of the RCF test are in closer agreement with those of the TPI. A similar view was also expressed by Ruge (1956).

The nature of the antibody detected by the test was studied by Sequeira (1959) who found that both thermolabile and thermostable fractions of syphilitic antibody are responsible for this reaction.

2. Reiter's fluorescent treponemal antibody test (RFTA)

Scott et al. (1961) suggested the use of Reiter's strain

as an antigen for the immune fluorescent test as they consider the strain to be antigenically similar to Nichols strain and easily cultivable in vitro.

They used the same technique as was used in the FTA-200 test but the antigen was prepared by suspending it in sodium hypochlorite-saline solution to prevent clumping and deformity of the organisms. The test was carried out on a number of TPI positive sera and on those from healthy individuals and blood donors along with the FTA test.

As regards the sensitivity of the RFTA test on syphilitic sera results were parallel with the FTA but were more sensitive in a few instances. Specificity of the test was confirmed in the healthy individuals and in the biologically false positive reactors where similar results were found with the FTA test except in one case of a non-specific reactor where the RFTA test showed reactivity in the absence of an FTA reaction.

From the results of their study they proposed further evaluation of the test as a possible substitute for the FTA test, which may replace the TPI test in the near future.

C. TEST WITH PROTEIN FRACTION OF VIRULENT TREPONEME

Treponema pallidum complement fixation test (TPCF)

To increase the sensitivity and specificity of the treponemal antigen used for the complement fixation test, Portnoy and Magnuson (1956), prepared an antigen from virulent T. pallidum by removing the lipid fractions and then extracting an active antigenic protein constituent by a solution of sodium desoxycholate, sodium citrate and sodium chloride. A similar attempt was made earlier by Saurino and Delamater (1952) by using hydrochloric acid for extraction. Portnoy and Magnuson (1955) used the Kolmer technique (1/5 vol.) in performing the test. After a proper evaluation of the test they came to the conclusion that the TPCF shows a close agreement with the TPI in biologically false positive reactors suggesting a high specificity. In primary and secondary syphilis the TPCF showed a high percentage of reactivity like the STS where the TPI gave a lower incidence of reactions, whereas in presumed latent and late syphilis the TPCF test showed a lower incidence of reactions as compared with both the TPI and STS. They also found an excellent reproducibility of the test; but this finding

was contradicted by Browne et al. (1959) who found that the TPI test is more reproducible than the TPCF test particularly when weak reactive sera were examined.

Price and Whelan (1957) mentioned the disadvantages of the test from its practical aspect as the preparation of antigen is lengthy and difficult, the yield is poor and the antigen cannot be stored for a long time. To circumvent the difficulties, Portnoy (1959) modified the technique with a small volume of antigen called the TPCF 50 test, the results of which showed a closer correlation with those of the standard technique. Portnoy and Magnuson (1956) also claimed that the antigen could be stored for 7 months without significant loss of its activity.

The nature of the antibody, detected by the TPCF test, was studied by different workers. McLeodand Stokes (1955) demonstrated two types of agglutinating antibodies in the syphilitic serum: one called reagin agglutinating antibody. They found a possible relationship between TPCF antibody and the non-reagin agglutinating antibody by the results of the inhibition test. Portnoy and Magnuson (1955) suggested that the TPI and TPCF antibodies were not identical as was found by the inhibition test in vitro and differential susceptibilities of the antibodies to heat. Sequeira (1959) considered the possibility of detection of the thermolabile and thermostable

(Immobilisin) antitreponemal antibodies by the TPCF test.

D. TEST WITH PROTEIN FRACTION FROM CULTIVABLE REITER'S TREPONEME: REITER'S PROTEIN COMPLEMENT FIXATION TEST (RPCF)

Fractionation and isolation of the antigenic constituents from Reiter's treponeme by D'Alessandro et al. (1949; 1953) led to the possibility of the introduction of the RPCF test in syphilitic serology. The authors isolated protein and carbohydrate fractions and two lipoids, one of which reacts with the reagin antibody, and is detected normally by the STS using the lipoid or cariolipin antigen. The carbohydrate fraction appeared to be relatively unimportant and most attention was paid to the thermolabile protein fraction, which was prepared by disruption of the treponemes by cryolysis or ultrasonic waves and by precipitation with increasing strengths of ammonium sulphate solution followed by dialysis. The final product could be used at a fairly high titre in the complement fixation test and wasnot found to be anti-complementary in marked contrast to the suspensions of the intact organisms. The authors found that the antigen gives positive reactions with a high percentage of sera from patients suffering from syphilis and yaws.

De Bruijn (1956) tested the sera with this antigen in parallel with the TPI test and showed that the RPCF test was both highly sensitive and specific. Subsequent reports (Cannefax and Garson, (1957); Rein et al., (1958); Miller et al. (1958); Foster et al., (1958); Sequeira, (1959); Wilkinson and Johnston, (1959))confirmed this finding. Foster et al. (1959) found that the test is more sensitive that the TPI test in early primary syphilis whereas it remains positive for a long time even in treated cases.

The nature of the antibody, detected by the RPCF test, was studied by different workers. D'Alessandro and Dardanoni (1953) found that the RPCF antigen can detect the presence of antitreponemal antibodies, different from antilipid antibodies, in syphilitic sera. However, they also commented that the antibody involved in the RPCF test does not appear to be the same as that responsible for the immobilisation of the treponemes in the TPI test, because an antibody produced in rabbit against RPCF antigen does not immobilise T. pallidum. Sequeira (1959) was of the opinion that the RPCF test could detect only the thermolabile antitreponemal antibody, quite distinct from the immobilising antibody (Immobilisin) detected by the TPI test.

Despite the advantages of the Reiter treponeme as an easily available source of antigenic material, Vaisman et al. (1958) thought that antigen extracted from the virulent treponemes (Nichols strain) may show more specific results due to the antigenic similarity with the causative organisms. They compared the results of the tests performed with protein antigens extracted from Reiter's and Nichols strains using the TPI as a reference test and found that the protein extracted from virulent organisms has a higher specificity and sensitivity than that made from the cultivable strain although sharing of a common antigen has been reported by Cannefax and Garson (1959).

The introduction and use of a large number of treponemal tests to replace the TPI test puzzled the serologists for a long time as proper evaluation of such tests was not carried out thoroughly until 1957. In 1957 the U. S. Public Health Service carried out a large-scale evaluation, known as the Serology Evaluation and Research Assembly (SERA) 1956-57. This evaluation included all the newer treponemal tests (except the Reiter's fluorescent treponemal tests) along with the TPI, together with the old complement fixation and flocculation tests with cardiolipin and lipoidal antigens.

The result of the evaluation showed a promising future for the Reiter's protein complement fixation (RPCF) and fluorescent antibody (FTA) tests regarding their sensitivity and specificity although the older serologic tests with cardiolipin antigen had a prominent place in the report.

STUDIES OF SERUM PROTEIN IN SYPHILIS

Along with the discovery of newer treponemal tests to replace the TPI test, extensive researches were carried out on the serum protein characteristics of syphilitic and biologically false positive individuals with a view to solving the problem of non-specific reactions in an easier way with the same lipoidal antigen. Serum protein (globulin) was fractionated into its different components (gamm, beta, alpha) either by salt precipitation or by elution from paper strips after electrophoresis. The serologic reactivity of the different globulin fractions from both true and false positive reactors, was tested with lipoidal antigen with some hope of finding a difference in the reactivity pattern.

Most of the studies on the serum protein in "biologically false positive reactors" were carried out after the discovery of the TPI test (as this test was found to be the standard one to discover such reactors) though the reagin activities of the different serum protein fractions were

studied earlier.

HUMAN SYPHILIS

Soon after the Wassermann reaction was introduced and other flocculation and complement fixation tests appeared a number of studies (Noguchi, 1909; Mackie, 1923, 1926; Gilmour, 1924; Eagle, 1937) were made on syphilitic sera in an effort to determine in which fraction or fractions the reagin occurred. In general all workers were in agreement with the fact that the reagin was present in the globulin fraction and was not present in the albumin fraction.

Cooper and Atlas (1943) showed a characteristic and significant alteration of the serum protein in all stages of syphilis. An increase of globulin component was found to be associated with a decrease of concentration of albumin, thus maintaining the total protein concentration within normal range. Later on Cooper (1944) successfully carried out the serologic tests on the five different fractions of syphilitic sera after precipitation with ammonium sulphate and subsequently confirmed the fractions by electrophoretic study. Serologic reactions with the Kahn standard and Wassermann tests showed activity in those fractions where the beta and gamma globulin were present.

Davis et al. (1945) studied the character of the Wassermann antibody after separation of the globulin fractions in the Tiseleus apparatus, by ultra-centrifugal concentration of whole syphilitic serum and electrophoretically separated gamma globulin. Serologic tests on the serum fractions showed the Wassermann antibody to have a mobility intermediate between the beta and gamma globulin, whereas ultra-centrifugal concentration showed that a portion of antibody has a higher molecular weight than the bulk of globulin with a sedimentation constant of 19 compared with 7 svedbergs.

Merklen and Berthaux (1952) considered the positivity of serologic reactions to be linked with a certain protein (globulin) constituent and not to be the property of the patient's serum suffering from syphilis. Reagin responsible for the serologic activity of syphilitic sera constitutes only a small quantity of the immunologically differentiated globulin and is mixed with many other globulins. They considered that the mobility of reagin is between $gamma_1$ and $gamma_2$ globulins. The stimulus for the production of this reagin is a lipid hapten and the sharing of a common antigenic structure between T. pallidum and crude lipoid antigen explains the reactivity of the latter with syphilitic serum.

Laurell (1955) showed the association of reagin with gamma globulin in the primary cases of syphilis whereas in the secondary stage, reagin migrates with both $beta_2$ and gamma globulins. In late syphilis she demonstrated it in $beta_2$ globulin. Nurolone (1957) found the reactivity of the antitreponemal antibody in gamma globulin whereas the antilipoid antibodies were detected in beta globulin. Ottolenghi (1957) could not find any serologic activity outside gamma globulin in syphilitic sera as $alpha_1$, $alpha_2$ and beta globulin fractions were pure though there was some overlap between the fast gamma globulin and slow beta globulin fractions. The author definitely demonstrated the separate existence of the flocculating and the complement fixing antibodies but failed to conclude the existence of an antibody as globulin as he thought that there may be some other substance travelling electrophoretically with the globulin fraction.

Nicolou and Badanoiu (1957) observed the increase of the serum globulin level in primary syphilis which reaches its highest peak in the secondary stage. In adequately treated cases, even with persisting serologic reactions, the electrophoretic pattern was normal in contrast with untreated or inadequately treated cases where hyperglobulinaemia

was a constant feature.

Laurell and Lindau (1957) found the presence of reagin in the gamma globulin in the initial stages of syphilis and its migration to $beta_2$ globulin as the disease advanced. They used paper electrophoresis for the fractionation of serum. Wolf (1957) noticed the initial abnormality of the electrophoretic pattern in a fair number of latent syphilis cases with a consistent change of an increased gamma globulin fraction in untreated syphilis which tends to become normal after treatment. He considered the electrophoretic abnormality to be due to increased activity of the R. E. system as the tests for paranchymatous liver disease were found to be normal.

Laurell and Hederstedt (1958) carried out the TPI and WR after elution of the different protein fractions from electrophoretic paper strips. They found the migration of the TPI antibody with the gamma globulin in contrast to Wassermann reagins which were located in gamma and $beta_2$ globulin fractions showing two distinct peaks but could not explain the relationship between them. Ottolenghi (1958) found the presence of antibodies in the gamma fraction in recent human syphilis which shifted to beta globulin in the secondary stage and to alpha globulin in the tertiary stage.

He also observed an increase in the reactivity of beta globulin with a reciprocal change in the gamma fraction within a few weeks after treatment. Gallego (1959) was of the opinion that the decrease of the A/G ratio is due to an increase in the gamma globulin fraction but an increase of globulin does not always show a parallelism with the intensity of serologic reaction.

EXPERIMENTAL RABBIT SYPHILIS

Kummel (1955) considered that serologic reactivity primarily depends on the gamma globulin component as he found a definite correlation between the rise and fall in titre of the W. R. on the one hand, and increase or decrease of gamma globulin fraction on the other without any deviation of alpha and beta globulin from their normal range.

STUDIES OF SERUM PROTEIN IN BIOLOGICALLY FALSE POSITIVE REACTORS

Coburn and Moore (1943) observed the presence of Wassermann reagin (after electrophoretic separation) in $beta_2$ and gamma fractions in the sera from false positive reactors suffering from systemic lupus erythematosus. As similar reactive fractions were also found in the sera from syphilitic individuals, they could not find any difference

between the true and false positive reactors by this method. Though the electrophoretic mobility was the same in both cases, the likelihood of similarities in other respects was not accepted by the authors.

Moore and Mohr (1952) noticed an occasional elevation of serum globulin with the shift of the A/G ratio from cases of biologically false positive reactors after exclusion of renal and hepatic functional disorders. On the other hand, Stout (1952) from Central America could not find any significant correlation between the false positive reaction and serum protein content.

Lindau and Laurell (1952) used paper electrophoresis to differentiate reactive fractions in syphilitic serum from a false positive reactor but were unable to solve the problem. Later Laurell (1955) repeated the same experiments and was convinced that lipoidal antigen could not be used for differentiation of reactivity in serum fractions from true and false positive reactors as similar reactivity was found in the same fractions (gamma and $beta_2$ globulins) in both types of cases. However, she classified biologically false positive sera into three types as reagin activity was associated with gamma globulin, with $beta_2$ globulin or with both the fractions.

Moore and Lutz (1955) considered the chronic biologically false positive reaction to be due to production of abnormal globulin (dysgammaglobulinaemia). They found the abnormality of the protein flocculation tests in a fair number of cases after the exclusion of derangement of liver function by Bromsulphthalein excretion and serum alkaline phosphatase determination. The authors were also of the opinion that the type of abnormality of protein flocculation tests or of serum globulin was not found with regularity in patients with syphilis.

Kostant (1956) could not support the view of alteration of serum protein fractions as an etiological factor in biologically false positive reactions. He argued that though the diseases like systemic lupus erythematosus and lymphgranuloma venereum are associated with hyperglobulinaemia and biologically false positive reactions, other diseases like multiple myeloma show hyperglobulinaemia without any manifestation of such reaction. Thus many false positive reactions are associated with normal protein values whereas others with altered serum protein values do not manifest biologically false positive reactions.

Miller et al. (1957) observed the abnormalities in caphalin flocculation, thymol turbidity and other tests indicating protein abnormalities in a large number of sera showing biologically false positive reactions without any clinical signs of other illness. However, Cannon (1958) could not find any abnormality in distribution of protein in his electrophoretic study on a few sera from congenital biologically false positive reactors.

Recently Laurell and Malmquist (1961) used a "DEAE-Cellulose" column for the separation of serum fractions (Peak I, II and V). Peak I and II contained gamma globulin of the 7s variety whereas Peak V showed the character of the 19s type (macroglobulin) although the purity of these fractions was marred by overlapping with the other serum fractions. Studying the reagin activity on the fractionated sera from normal, syphilitic (primary, secondary and tertiary) and biologically false positive individuals the authors came to the conclusion that normally occurring reagin is a macroglobulin (Peak V) which participates in biologically false positive reactions along with the 7s variety (Peak I). The macroglobulin type of reaction is predominant in the primary syphilis and with the progress of the disease to secondary and tertiary stages, reagin of low molecular

types (belonging to Peak I and II) appear although activity of Peak I may disappear in the tertiary stage.

SUMMARY

The history of the developments in the serology of syphilis has been conducted at considerable and perhaps wearisome length. The excuse, if any were needed, is due to the non-specificity of the serologic reaction with lipoidal antigen and as a consequence a large number of tests have been developed since its inception. Earlier attempts to solve the problem were made in an unplanned way without any fundamental approach to the basis of the false positive reaction. Later on attention was focussed on an impurity in the antigenic material and resulted in the discovery of the synthetic cardiolipin antigen. But unfortunately these attempts did not solve the problem. With the introduction of the TPI test the problem of specificity was solved to a great extent but technical diffuclties restricted its use. So research progressed to simplify the use of treponemal antigen in different ways either as dead antigen or by using cultivable strains of treponemes. This led to a newer group of tests though only a few (RPCF and FTA) have shown promising results (as mentioned in the SERA report

(1956-57) published by U.S. Public Health Service).

Simultaneously, attempts were made to solve the problem of non-specificity by finding out the serologic activity of different serum protein fractions from syphilitic and biologically false positive individuals so that the old lipoidal antigen could differentiate between them. Different methods such as salt precipitation and electrophoretic separation were used to fractionate the serum proteins but none were suitable for subfractionation of gamma globulin (gamma $_1$ and gamma$_2$) and overlappings of different fractions were found in salt precipitation. Such studies on protein fractions did not show any definite result because similar reactivity was found in the same fraction of serum (gamma and beta$_2$) in both types of cases; reactivity of reagin was also manifested in the alpha fraction in the tertiary stage of the disease.

In the past, reagin activity in fractionated sera was determined only by the Wassermann reaction and the reactivity of serum fractions was studied in different stages of human syphilis without any attempt to study it systematically on sera from infected animals. The introduction of "DEAE-Cellulose" column chromatography in this field by some workers during the course of the work reported in this thesis,

helped in subfractionation of gamma globulin (gamma$_1$ and gamma$_2$) to a certain extent but could not solve the problem of nonspecific reactions as a similar reactivity pattern was found with fractions from both true and false positive sera.

ORIGINAL INVESTIGATIONS

ORIGINAL INVESTIGATIONS

The primary purpose of the present investigation is to compare and contrast the sensitivity and specificity of the newer serologic tests using treponemal antigens (RPCF, FTA, RFTA) along with the TPI and non-treponemal tests with crude lipoid and cardiolipin antigens (WR, KT and VDRL).

The second purpose is to study the serologic reactivity of different serum protein fractions from experimental rabbit syphilis (at regular intervals after infection), different stages of human infection, normal healthy adults and biologically false positive reactors. It may be possible from such a study to determine the relationship between the reactivity of different serum protein fractions with different types of antigen and consequently to solve the problem of biologically false positive reactions.

The recent introduction of column chromatography with anion exchange resin has improved and simplified the method of fractionation of serum protein. Sera were fractionated by means of "DEAE-Sephadex" (Pharmacia, Uppsala, Sweden) column - an anion exchange resin - in this study.

The investigations and results presented here are described in the following parts:

PART I: Study of sensitivity and specificity of serologic

tests using newer treponemal antigens (Reiter protein complement fixation test, Fluorescent treponemal antibody test, Reiter fluorescent treponemal antibody test).

(a) Study of sensitivity in -

(i) experimental rabbit syphilis

(ii) early primary human syphilis

(b) Study of specificity in biologically false positive reactors from -

(i) healthy blood donors

(ii) confirmed cases of rheumatoid arthritis

(iii) miscellaneous diseases

PART II: Study of serologic reactivity of serum protein fractions in -

(a) experimental rabbit syphilis

(b) normal healthy persons

(c) natural infection at various stages

(d) biologically false positive reactors

PART I

Study of sensitivity and specificity of serologic tests.

Chapter II

METHODS

The serologic tests included in this evaluation study were those of recently introduced treponemal antigen tests, namely - Reiter protein complement fixation test (RPCF), Fluorescent treponemal antibody test (FTA) and Reiter treponemal fluorescent antibody test (RFTA). Inclusion of non-treponemal tests like Wassermann reaction (Price's technique), Kahn standard test and cardiolipin slide flocculation test (VDRL) were due to their popularity, simplicity of technique and for their participation in biologically false positive reactions.

Treponema pallidum immobilisation test (TPI) was also included as it was considered to be the basic standard test for proper evaluation.

NON-TREPONEMAL TEST

I. Wassermann Reaction (Price's technique) (Price, 1948, 50).

Reagents:

1. Saline 0.9% NaCl (A. R. Reagent grade)
2. Complement-Fresh pooled guinea pig sera.
3. Sensitised sheep cells - Formolised sheep cells (Burroughs Wellcome & Co. London) were washed three times with 0.9% saline and were packed by centrifugation

after last supernatant becamedear. A 5% suspension in saline was made from the packed deposit and after thorough mixing 1 ml. was placed in a haematocrit tube and was centrifuged at 3,000 r.p.m. for 10 minutes. The height of the column of packed cells was read off and the amount of saline to be added to give a 5% suspension was calculated and mixed with the original suspension. A solution of amboceptor (Burroughs Wellcome & Co. London) containing 10 M.H.D. per unit volume was prepared according to the titre of the serum. Equal volume of 5% cell suspension and 10 M.H.D. amboceptor solution were then mixed rapidly by pouring from one container to another about six times and the resulting $2\frac{1}{2}$% suspension sensitised with 5 M.H.D. amboceptor was incubated for half an hour in a waterbath at 37°C and the container was shaken occasionally to resuspend the cells.

4. Wassermann Antigen.

This was obtained from the V.D. Reference Laboratory, Whitechapel, London. Three parts of alcoholic extract of heart muscle were mixed with two parts of a 1% alcoholic solution of cholesterol. The resulting mixture was diluted with saline (0.9%) according to its optimal titre and was left to ripen at room temperature for 20 minutes before use.

5. Sera

These were inactivated for 30 minutes at 56°C. Serum fractions were also inactivated in a similar way.

Measurement of volumes

The unit volume used in the test was .11 ml. This was dispensed by a teated Pasteur pipette graduated to deliver this volume. Sera were dispensed with Pasteur pipette delivering drop of serum equal to .022 ml.

Complement titration

Five horizontal rows of six tubes were set out in a Wassermann rack and the tubes in each row were numbered 1/20, 1/30 .... 1/70, to correspond to the dilutions of complement tested. The reagents were distributed as shown in table.

TABLE I - LAY OUT OF COMPLEMENT TITRATION

Row	Complement 1/20 - 1/70	Negative serum	Positive serum	Anti-gen	Saline
1	1 vol.	-	-	-	2 vols.
2	"	-	-	1 vol.	1 vol.
3	"	1/5 vol.	-	"	"
4	"	1/5 vol.	-	-	2 vol.
5	"	-	1/5 vol.	-	"

After incubation at 37°C for an hour, 1 vol. of sensitised cell suspension was added to each tube and after thorough shaking the tabes were reincubated for 30 minutes at 37°C.

Reading:

The control dose of complement for the serum control tubes of the test was given by the highest dilution of complement in rows 4 and 5 which showed sparkling lysis.

The Diagnostic dose of complement was based on the highest dilution of row 3 which showed sparkling lysis. This was taken as 1 M. H. D. and 1.25 M. H. D. was used for the diagnostic tubes of the test.

Test proper

Qualitative test:

Two tubes were set out for each specimen

	Serum	Serum fraction
Diagnostic Tube	1/5 vol. serum	1 vol. serum fraction
	1 vol. antigen	1 vol. antigen
	1 vol. saline	1 vol. saline
	1 vol. diagnostic dose of complement	1 vol. diagnostic dose of complement

	Serum	Serum fraction
Control tube	1/5 vol. serum	1 vol. serum fraction
	2 vol. saline	2 vol. saline
	1 vol. control dose of complement	1 vol. control dose of complement

After incubation for one hour at 37°C in water bath, 1 vol. of sensitised cells was added to each tube and racks reincubated for a further 30 minutes at 37°C and readings were taken.

Reading: Complete haemohysis in both tubes was read as a negative reaction. No haemolysis in the diagnostic tube with complete haemolysis in the control tube was read as a positive reaction. Partial haemolysis in the serum diagnostic tube and complete haemolysis in the control tube was read as a weakly positive reaction. Reading was made as anti-complementary when any particular sample of serum or fraction failed toshow complete haemolysis in the control tube.

Quantitative test:

In this method doubling dilutions of the serum and serum fractions in 0.9% saline from 1 in 5 to 1 in 320 were

used. They were set up as follows:

	Serum	Serum fractions
Diagnostic tube	1 vol. serum diluted 1/5, 1/10....	1 vol. serum fraction diluted 1/5, 1/10....
	1 vol. antigen	1 vol. antigen
	1 vol. diagnostic dose of complement	1 vol. diagnostic dose of complement
Control tube	1 vol. serum diluted 1/5	1 vol. serum fraction diluted 1/5
	1 vol. saline	1 vol. saline
	1 vol. control dose of complement	1 vol. control dose of complement

The sensitised red cells were added in the same manner as in the test proper and the incubation time was also the same.

Reading: The titre was taken as the highest dilution of serum or serum fractions showing definite inhibition of lysis.

Batch control: Pooled positive and negative sera used for complement titration, were included in each batch of tests.

II KAHN STANDARD TEST

Reagents

1. Kahn standard antigen was obtained commercially from Burroughs Wellcome & Co., London.
2. Saline. 0.9% NaCl (A. R. Reagent grade) in distilled water.

Preparation of serum

Sera were inactivated for 30 minutes at 56°C. Serum fractions were also treated similarly before test.

Preparation of antigen suspension

The necessary amount of saline for a given amount of antigen was measured in an antigen suspension vial (flat bottom, cylindrical glass tube without rim; capacity - 5ml.) according to titre of the antigen (labelled on antigen bottle) and the necessary amount of antigen was measured into a second vial. Saline was poured into antigen and without stopping they were mixed back and forth 12 times avoiding complete drainage during mixing period. The antigen suspension was then allowed to stand for 10 minutes at room temperature before use.

Qualitative test:

(a) Serum

0.15 ml. of inactivated serum was added to each tube of all

the rows of Kahn rack by a Pasteur pipette delivering drop of serum equal to 0.05 ml.

Antigen suspension was thoroughly shaken to disperse the lipid aggregates before use. 0.0125 ml. of antigen suspension was then added to the serum of each tube in the last row by a dropping pipette delivering the same amount per drop. Similarly .025 ml. and .05 ml. of suspension were placed in the tubes of the second and first row respectively.

The rack was shaken by hand for 10 seconds after antigen suspension was added to all the tubes in the rack.

The rack was then placed in an incubator (37°C) for 7 minutes and was subsequently shaken in a Kahn shaker (270 oscillations per minute) for 3 minutes.

After completion of shaking 1.0 ml. of 0.9 per cent saline solution was added to each tube of the front row and 0.5 ml. of the same solution was added to each tube of the middle and back rows.

The rack was then shaken gently by hand to mix the content of the tubes.

Positive serum, negative serum and saline solution were employed in each batch of test using sameantigen suspension.

Reading of result:

Microscopic mirror (concave) and a table lamp were used to view the image of tube contents and the degree of flocculation.

Recording of results:

Degree of flocculation was determined with the following outline:

4+ ..... Relative large floccules

3+..... Mediumsized floccules

2+ ..... Fine floccules easily distinguishable

1+ ..... very fine floccules

± ..... extremely fine floccules just distinguishable

- ..... an opalescent medium free from visible floccules.

Reporting of results:

The results were reported as positive, weakly positive and negative according to the table.

TABLE II RECORDING OF RESULTS

Sum of pluses in the 6-tube reading	Final result
22 to 24	Positive 4+
16 to 21	Positive 3+
10 to 15	Positive 2+

Table II (Cont'd)

Sum of pluses in the 6-tube reading	Final result
5 to 9	Weakly positive 1+
4	Weakly positive ±
3 or less	Negative

(b) Serum fractions

Serum fractions were tested similarly and reactivity was judged in a similar way.

Quantitative test:

Sera and serum fractions were diluted with 0.9% NaCl solution in proportion of 1:2, 1:4, 1:8, 1:16, 1:32 and higher when necessary.

0.15 ml. of each diluted serum or fraction was placed in each tube of the row by means of a dropping Pasteur pipette (delivering 0.05 ml. per drop) starting from the highest dilution.

0.0125 ml. of antigen suspension (prepared as described under qualitative test) was then added to the sample of each tube by means of antigen dropping pipette (delivering 0.0125 ml. per drop).

After shaking the rack by hand for 10 seconds, it was incubated and shaken in a Kahn shaker in the same manner as

in the qualitative test. 0.5 ml. of 0.9% NaCl was added to each tube and results were recorded and reported as in the test proper. The titration end point was determined as the highest dilution of serum or fractions in which a 4+, 3+ or 2+ reaction was observed.

III VDRL SLIDE FLOCCULATION TEST

Reagents:

1. Antigen: This was obtained from Burroughs Wellcome & Co. London, in sealed glass ampoules in different batches.
2. Saline: Buffered saline containing 1% sodium chloride was obtained from the same source along with each lot of antigen ampoules.

   0.9% saline was prepared by adding 900 mgm. of dry sodium chloride (A.R. Reagent grade) in one litre of distilled water.

Preparation of serum

Sera were heated at 56°C in a water bath for 30 minutes. Serum fractions were also inactivated in a similar way.

Preparation of slides

Clean 2" x 3" glass slides were used after paraffin

rings were made by transferring melted paraffin to the slides by means of a metal ring (diameter - 14 m.m.).

Preparation of antigen emulsion

0.4 ml. of buffered saline solution waspipetted to the bottom of a 30 ml. glass stoppered bottle. 0.5 ml. of antigen (from lower half of a 1.0 ml. pipette graduated to the tip) was then added drop by drop, but rapidly, so that approximately 6 seconds were allowed for each 0.5 ml. of antigen. The bottle was rotated continuously on a flat surface during the period of addition of antigen and was continued for a further 10 seconds. 4.1 ml. of buffered saline was then added to the mixture from a 5 ml. pipette. The mixture was then shaken thoroughly (30 times) for 10 seconds and was used for the test.

Qualitative test

Serum 0.05 ml. of inactivated serum was delivered into one ring of a paraffin ranged glass slide by means of a Pasteurpipette delivering 0.05 ml. serum per drop.

One drop (1/60 ml.) of antigen emulsion was then added to each drop of serum by means of another measured dropping pipette delivering the same amount of antigen per drop.

The slide was then rotated by hand (which circumscribed 2 inches diameter circle with a speed of 120 times

per minute) for 4 minutes. The tests were then read immediately after rotation. Serum controls (positive and negative) were included during a testing period to ensure proper reactivity of antigen emulsion.

Reading and reporting of test results

Tests were read microscopically, with low power objective, at 100 x magnification. The antigen particles appeared as short rod forms at this magnification. Aggregation of these particles into large or small clumps was interpreted as degrees of reactivity.

Reading	Report
No clump or very slight roughness	Negative
Small clumps	Weakly positive
Medium and large clumps	Positive

To avoid zonal reaction all sera producing weakly positive results in qualitative test were retested using quantitative procedure before reporting VDRL slide test. Thus when a positive result was obtained in some dilution of a serum that produced only a weakly positive result as undiluted serum, the report was made as positive.

Serum fractions

Serum fractions were tested similarly and reactivity

was judged in a similar way.

Quantitative test

Inactivated sera and serum fractions were diluted with 0.9% NaCl solution in proportion of1:2, 1:4, 1:8, 1:16, 1:32 and higher when necessary. Dilutions were made on a series of paraffin rings (inscribed on the glass slide) by means of 0.05 ml. standard Pasteur pipette.

One drop (1/60 ml.) antigen emulsion was then added to each drop of diluted preparation by the measured antigen dropping pipette and the slide was rotated by hand similarly as described in qualitative test.

Reading and reporting of test result

The tests were read microscopically at 100 x magnification as described under qualitative test procedure. The result was reported in terms of highest serum dilution that produced a positive (not weakly positive) result in accordance with the following example.

Serum dilutions					Report
1:2	1:4	1:8	1:16	1:32	
P	P	WP	N	N	positive 1:4 dilutions or 4 dils.

P = positive, WP = weakly positive, N = negative.

TREPONEMAL TESTS

I. TREPONEMA PALLIDUM IMMOBILISATION TEST (TPI)

Principle

When living T. pallidum are incubated with syphilitic serum in the presence of complement they are immobilised and killed. With normal non-syphilitic serum and complement they remain motile.

Maintenance of strain of treponemes

The pathogenic Nichols strain (obtained from the V. D. Reference Laboratory, Whitechapel, London) of T. pallidum was used. Two adult male rabbits were inoculated intra-testicularly in each week with about 50×10^6 treponemes (present in 1 ml. inoculum) in each testicle. One of these rabbits was injected intramuscularly with 6 mgm. of cortisone acetate daily for 7 days while the other was kept without any treatment. Orchitis developed within 6 - 9 days in the untreated rabbit whereas little swelling was manifested in the cortisone treated rabbit. The cortisone treated rabbit was killed routinely on the eighth day of inoculation for the maintenance of the strain and also for antigen suspension in the test proper. The untreated inoculated rabbit was mainly acting as a safeguard against the loss of the strain.

A regular supply of rabbits (without any differentiation of species) was obtained from a recognised dealer in Edinburgh. Initially when the size of such rabbits was small, difficulty was experienced in propagation of the strain as the injection of 1ml. of the inoculum into the testis was usually followed by some leakage from the site of the injection. This prevented the growth of a sufficient number of treponemes to maintain the strain in the next batch of rabbits. But this difficulty was overcome when injections were done properly by inserting the needle deeply into the body of the testis with consequent retention of the whole inoculum.

Serum

Blood samples were collected aseptically in sterile vials and sera were stored in the deep freeze at -20°C without any preservatives. These were thawed and inactivated for 30 minutes at 56°C before testing. When samples of blood were collected from penicillin-treated infected rabbits, its toxic effect was neutralised by the incubation of serum with penicillinase (20 units per ml. of serum) before testing.

Serum fractions were also inactivated in a similar way.

Complement

Active complement (C+) Blood samples were collected from a large number of male guinea pigs by cardiac

puncture. Sera were pooled and separated. Complement thus prepared was sterilised by filtration with a sintered glass filter (bacteria free) before storage at -20°C. The complement kept at this temperature was usable for about a month.

Inactive complement (C-) Active complement was inactivated for 30 minutes at 56°C just before use in the test.

Survival medium

Treponemes survived well and maintained their characteristic motility in this medium. Redistilled water was used for preparation of all solutions. The medium was made up in bulk and was of the following composition:

5% Bovine Albumen, Fraction V in saline			
pH adjusted to 7.0			100 ml.
Phosphate buffer, pH 7.1			
0.1 M. Na_2HPO_4	50 ml.	Mixture	31.3 ml.
0.1 M. KH_2PO_4	25 ml.		
1.23% reduced glutathione in saline			6.25 ml.
0.63% l - cysteine hydrochloride in saline			6.25 ml.
1.0% Sodium pyruvate in saline			3.13 ml.
1.5% Sodium thioglycollate in dist. water			12. 0 ml.
1.26% Sodium bicarbonate in dist. water			11.4 ml.
Ultrafiltrate of beef serum			25. 0 ml.
pH of medium adjusted to 7.0			

Sterilised by sintered glass (bacteria free) filtration and was distributed in 20 ml. amounts in screw capped vials and was stored at -20°C. At this temperature the media worked satisfactorily for about six weeks.

Preparation of treponeme suspension

20 ml. of the survival medium was placed in an extraction flask and the air was replaced by a mixture of 95% N_2 and 5% CO_2. This was obtained commercially from the British Oxygen Co. Ltd. After gassing, the flask was shaken gently at 35°C for about an hour so that the medium could be saturated with the gas.

The cortisone-treated rabbit was killed and the testicles were removed aseptically. After cutting into thin slices, so as to rpovide as large a surface area as possible, they were placed in the extraction flask with the equilibriated medium. The flask was then gassed and shaken gently at 35°C in a waterbath shaker until a sufficiently dense treponeme suspension was obtained (about 10-15 per x 400 magnified field). The suspension was then centrifuged gently for 10 minutes to deposit red cells and tissue debris and the supernatant was decanted off for use in the test. 1 mg. streptomycin sulphate was added per ml. of suspension to inhibit the growth of bacterial contaminants.

About 5 ml. fresh medium was added to the testicles in the extraction flask and after gassing this was shaken for $\frac{1}{2}$ to 1 hr. on a Kahn shaker. This gave a dense suspension of treponemes which, after centrifugation, was used to inoculate two other new rabbits. A rich inculum with a density of about 50×10^6 was the optimum level for getting a rapidly developing orchitis. About 1 ml. of this inoculum was used for each injection after inserting the needle deeply into the body of testis.

Setting up the test

This was carried out in sterile 10 x 1 cm. tubes of pyrex glass with loosely fitting aluminium caps. Reagents and sera were distributed with dropping pipettes, one drop = 0.05 ml.

Batch controls

Tube1	Trep. susp.	0.5 ml.	check on survival
Tube 2	Trep. susp.	0.3 ml.	
	C-	0.15 ml.	complement
Tube 3	Trep. susp.	0.3 ml.	control
	C+	0.15 ml.	
Tube 4	Trep. susp.	0.3 ml.	
	Negative serum	0.05 ml.	
	C-	0.15 ml.	Negative control of
Tube 5	Trep. susp.	0.3 ml.	sensitivity
	Negative serum	0.05 ml.	
	C+	0.15 ml.	

Tube 6	Trep. susp.	0.3 ml.	
	Positive serum	0.05 ml.	
	C-	0.15 ml.	Positive control of sensitivity
Tube 7	Trep. susp.	0.3 ml.	
	Positive serum	0.05 ml.	
	C+	0.15 ml.	
Tube 8	Trep. susp.	0.3 ml.	
	Unknown serum	0.05 ml.	
	C-	0.15 ml.	Test
Tube 9	Trep. susp.	0.3 ml.	
	Unknown serum	0.05 ml.	
	C+	0.15 ml.	

In quantitative tests each serum was diluted to three times and its multiple with normal saline (pH 7.0) and each diluted serum was tested qualitatively but only one control with C- was used with lowest diluted serum.

Serum fractions were put up for test in a similar way. After addition of the reagents the tubes were gently shaken to mix the contents and were placed in a vacuum desiccator along with an anaerobic control containing .45 ml. of medium with a few drops of methylene blue (1%). The air was removed by four seccessive exhaustions and replacements by the N_2 - CO_2 mixture and the tests were incubated for 18 hours at 35°C.

Reading of the tests

A loopful of the contents of each tube was transferred to a slide, a coverslip was applied and the preparation was

examined by darkground under a magnification of x 400. For the batch control 50 successive treponemes were counted and the percentage of motile organisms were estimated.

For the test sera counts on 25 successive treponemes were used but 50 treponemes were counted where the result was in doubtful range of immobilisation. From the percentages of motile treponemes in each pair of tubes with active and inactive complement the "specific immobilisation" was calculated.

$$\text{Specific Immobilisation (S. I.) as \%} = 100 \times \frac{(\%\text{ motile in c-tube}) - (\%\text{ motile in c+tube})}{(\%\text{ motile in C-tube})}$$

Criteria of positivity 0 - 19% S. I. = negative

20 - 49% S. I. = doubtful

50 - 100% S. I. = positive

In quantitative test the titre was expressed as the (final) serum dilution which immobilised 50% of treponemes. Doubtful reactions were disregarded when tested with serum fractions.

Check on residual complement

Before the negative result was accepted as valid the activity of complement in C+ tube was tested in all cases. After the readings were taken 0.15 ml. of veronal buffer (pH 7.2) containing Ca++ and Mg++ was added to each C+ tube following

by 0.2 ml. of a $2\frac{1}{2}\%$ sheep cell suspension sensitised with 5 M.H.D. amboceptor. The tubes were incubated at 37°C for 30 minutes and complete haemolysis determined the presence of active complement.

II FLUORESCENT TREPONEMAL ANTIBODY TEST

Antigen Treponeme (Nichols strain) suspension was prepared from the inoculum (testicular extract) which was left over after inoculation into rabbit testicles in TPI test. The inoculum used for the preparation of antigen was from the cortisone treated rabbit. The suspension was kept in the refrigerator (4° - 6°C) for a period of 7 days and the tissue particles were separated by low speed centrifugation at 2000 r.p.m. for a period of 7 minutes. Later on treponemes present in supernatant fluid was concentrated by high speed centrifugation at 4000 r.p.m. for 20 minutes and resuspended in the same fluid after discarding some portion of the supernatant so that the final concentration was adjusted to 50 to 75 organisms per high dry field. 1 mg. of streptomycin sulphate was added per ml. of suspension to inhibit the growth of bacterial comtaminants. Suspension so prepared remained stable in the refrigerator (4° - 6°C) for several months, but before being put in use was checked to ensure

that they did not show any non-specific staining with conjugates.

Sera

These were stored at -20°C and inactivated at 56°C for 30 minutes before testing. If retested they were again heated at 56°C for 10 minutes.

Serum fractions were also treated similarly before testing.

Buffered saline

NaCl. 6.8 gms., Na_2HPO_4, 1.48 gms., KH_2PO_4 0.43 gms. were dissolved in one litre of distilled water and pH was adjusted between 7.2 - 7.3.

Conjugates

1. Rabbit antihuman globulin conjugate - This was prepared from stock rabbit antihuman globulin sera (Coombs) obtained from Burroughs Wellcome & Co., London. Before using the sera for conjugation the potency was checked by a precipitation test with human gamma globulin. The globulin fraction of the serum was separated out by precipitation with saturated solution of ammonium sulphate in the cold after mixing with an equal volume of diluted antiserum (equal volume of antiserum and buffered saline, pH 7.2).

The precipitate was then washed once with half saturated ammonium sulphate by centrifugation and was dissolved in distilled water. Ammonium sulphate was driven out of the precipitate completely by dialysing against diluted buffered saline (buffered saline: distilled water - 1:20) and the protein content of the solution was determined.

The globulin fraction was then conjugated with fluorescein isothiocyanate (British Drug House, London) by method of Marshall et al. (1958) as follows:

Reagents

A. 1. Normal saline 10 ml.

2. Na_2CO_3 - 16.6 gms.

NaH_2CO_3 - 16.8 gms.

Distilled water 1000 ml. 3 ml.
pH - 9.0

3. Acetone (anhydrous and chemically pure) 2 ml.

-15 ml.

B. Globulin preparation (containing 1% protein)

-10 ml.

C. Fluorescein isothiocyanate (.05 mg. for each mg. of globulin)

-5.0 mg.

Reagents were mixed in a conical flask and the mixture was gently stirred overnight in a cool room (4° - 6°C) by a magnetic stirrer. The conjugate was then dialysed against 1000 ml. of diluted buffer saline (buffered saline: distilled water - 1:20) to drive out the excess fluorescein.

The conjugate was titrated by an optimal proportion procedure in the FTA test against a known positive human serum and was used at the highest dilution which showed a definite reactive reading with the greatest dilution of the control serum. The conjugate was stored in a refrigerator (4° - 6°C) after adding merthiolate solution (1:10, 000).

This conjugate was used for testing samples of human sera.

2. Sheep antirabbit globulin fluorescein conjugate - This preparation was obtained commercially from Sylvana Chemicals, Orange, New Jersey, U. S. A. The antiserum globulin was conjugated with fluorescein isothiocyanate and was preserved with merthiolate (1:10, 000) solution.

The conjugate was also titrated by an optimal proportion procedure in the FTA test against a known positive serum of rabbit, which was infected with Nichols strain of T. pallidum. The conjugate used in the test was the highest

dilution which showed a definite reactive reading with the greatest dilution of control serum.

Technique

Qualitative test

Two circles of 1 cm. in diameter were inscribed on microscopic slide with a diamond pencil and a 4 m.m. loopful of treponeme suspension was spread within the marked area and allowed to dry in an incubator (37°C) for 20 minutes. The slides were then fixed in acetone for 10 minutes.

0.03 ml. of a 1 in 200 dilution of serum in buffered saline was added to the fixed treponemes with a dropping pipette and the slides were incubated in a moist chamber for one hour at 37°C. (In case of rabbit serum undiluted serum was used for the qualitative test).

Excess serum was washed off gently with normal saline and the slides were soaked in two changes of buffered saline for 5 minutes in a Coplin jar.

After removing excess buffered saline, 0.03 ml. of specific conjugate, diluted to its titre in 2 per cent. Tween 80 in buffered saline was added and the slides were incubated for a further 30 minutes at 37°C in a moist chamber.

After excess conjugate had been washed off withnormal saline, the slides were again soaked for 5 minutes in two changes of buffered saline. The excess of buffered saline was removed by gently wiping out with dry gauze and the smears in circles were mounted in glycerene buffered saline mixture (Glycerine: buffered saline - 9:1).

Quantitative test

Each serum was diluted to 1 in 200 and its multiple in buffered saline and each diluted serum was tested qualitatively. In case of rabbit serum the dilutions used for the test were 1:10, 1:100, 1:200 and higher when necessary.

Serum fractions were put up for test in a similar way starting from 1:5 dil. but the qualitative test was performed only on the neat fraction.

Optical equipment

The ultraviolet source was a 250 watt high pressure mercury vapour lamp with a condensing lens. The light was passed through a trough of 5% copper sulphate and an excitation filter (B. G. 12). Observation was made by a darkground microscope with x 400 magnification under high power. An ocular filter, Ratner 2B, was used with the eye piece to prevent any residual ultraviolet light reaching the eye. The illuminating system was fixed to a

base board and the observation was made in a dark room.

Reading of result

The following scale was adapted for judging the intensity of fluorescence.

(a) ++++ very brilliant apple green fluorescence

(b) +++ brilliant fluorescence

(c) ++ definite fluorescence, not as marked as in (b)

(d) + faint fluorescence

(e) ± treponemes just visible but no green fluorescence

(f) o treponemes not visible

Grades (a), (b) and (c) were classified as positive and grades (d), (e) and (f) as negative. When no treponemes could be seen, the slide was checked by darkground examination with visible light to ensure that the organisms had not been accidentally removed from the slide during staining procedure. Strong positive, weakly positive and negative sera were included in each batch of tests.

III. REITER'S FLUORESCENT TREPONEMAL ANTIBODY TEST (RFTA)

Antigen

Reiter's strain of treponeme was cultivated at 35°C in screw-capped vials containing 25 ml. routine liver broth

thioglycollate glucose medium. A five-day old culture (dead) was obtained from the V. D. Reference Laboratory, Whitechapel, London and the antigen was prepared by concentrating the treponemes after centrifugation and washing it three times in normal saline. The final dilution of the antigen was made in normal saline and the concentration of treponemes was adjusted to 50 to 75 per high dry field (x400 magnification). It was stored in refrigerator (4^{o} - 6^{o}C).

Besides the antigen, other reagents used in this test were similar to those in the FTA test.

Technique

A qualitative test was carried out exactly in a similar way as that of the FTA test. The quantitative test on rabbit sera was carried out on neat serum and at different dilutions like 1:5, 1:10, 1;20, 1:40....., as the test was found to be less sensitive than the FTA test.

Results

The results were also recorded similarly to the FTA test after examination of slides under fluorescent microscope set up as for the FTA test (Fig. 1).

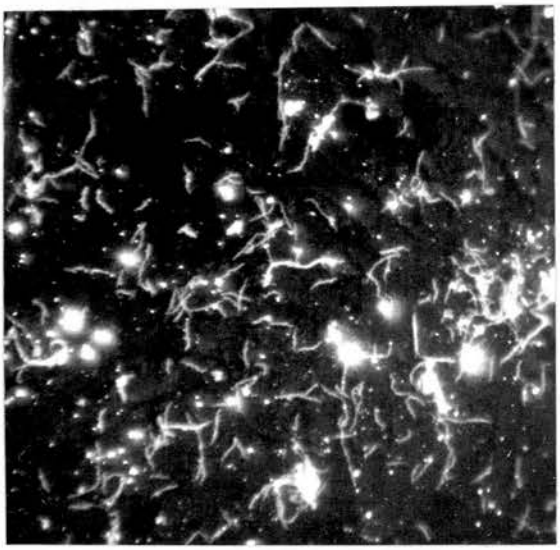

Fig. 1.

Reiter fluorescent treponemal antibody (RFTA) test. Smear of T. pallidum (Reiter strain) treated with positive rabbit serum and then fluorescent isothiocyanante-conjugated anti-rabbit-globulin x 400.

IV REITER PROTEIN COMPLEMENT FIXATION TEST (RPCF)

Antigen

The Reiter protein antigen is a highly purified lipid free protein antigen. This is prepared from T. pallidum (Reiter's strain) after disruption with ultrasonic vibrations. The protein fraction is then precipitated with ammonium sulphate solutionand is purified by dialysis.

The antigen was obtained from N. V. Organon, Oss, Holland (manufactured by the National Institute of Public Health, Utrecht, Holland) in lyophilised form inside a sealed glass ampoule with a titre of 1:80. The antigen was dissolved in 2 ml. of distilled water after heating gently for a few minutes in a 37°C water bath. The solution was then diluted with 78 ml. normal saline and was kept in the refrigerator (4° - 6°C). This diluted antigen could be used for indefinite periods if aseptic precautions were taken during dilution of the antigen.

The test was carried out by Price's technique (Price, 1948, 50) of Wassermann reactions as mentioned earlier.

The results were also reported in a similar way.

Chapter III

MATERIALS

Comparison of tests for sensitivity

Sensitivities of the different serologic tests were judged from their reactivities with the sera from two groups of cases.

1. Experimental rabbit syphilis
2. Early primary human syphilis

1. Experimental Rabbit Syphilis

Nine adult healthy male rabbits (6 - 8 months old) were chosen for infection. Their sera were tested by the different serologic methods before injecting them intratesticularly with Nichols strain of T. pallidum (1 ml. inoculum containing 50×10^6 organisms). The infection was confirmed by the presence of treponemes in the aspirated testicular fluid on the 8th day following injection.

The rabbits were then divided into two groups (treated and untreated).

Treated group (5 rabbits): Blood samples of 10 ml. each were collected aseptically from the ear veins in each week for the first 28 weeks and thereafter at biweekly intervals until the 40th week after which two such samples were taken at a 4-weekly interval. These rabbits were treated on the

12th week of infection by intramuscular injection of 30,000 units procaine penicillin daily for a period of 8 days. Quantitative serologic tests were performed on each serum sample until an end point was reached.

Untreated group (4 rabbits): 10 ml. of blood samples were collected from each rabbit at the same periodic intervals as the treated group starting on the 14th week from the day of infection. The collection of blood samples were delayed as it was presumed that all the rabbit sera in this group would behave in a similar way in their serologic reactivities as was found in the treated group in their pre-treatment periods. The quantitative serologic tests were performed on the sera as for the treated group of rabbits.

During the whole period of investigations each rabbit from both groups was fed daily with an extra diet composed of oat meal and bran containing 1/10th grain of powdered ferrous sulphate tablet to prevent anaemia from repeated blood loss.

2. Early Primary Human Syphilis

Specimens of 25 sera were obtained from a number of venereal disease clinics in England and Scotland. These sera were collected from confirmed cases of early primary

syphilis. Thepresence of treponemes in the primary sore, history of exposure and the clinical manifestations confirmed the diagnosis in such cases.

Different qualitative serologic tests (WR, KT, VDRL, RPCF, TPI, FTA-200, RFTA-200) were carried out with the same sample of serum and their relative sensitivities were compared.

In those sera where the FTA-200 test showed negative results, a quantitative FTA test was performed with the same sample in dilutions of 1:5, 1:10, 1:25, 1:50, 1:100. The titres of these sera were then compared with the results of a similar test carried out with 25 samples of sera from normal healthy adults using the same group of reagents. This comparative study was performed to reduce the dilution of serum used in the FTA-200 test so that the sensitivity of the FTA test (at a much lower dilution) could be increased in serologically non-reacting primary cases without showing any reactive result in the normal sera at such a dilution.

Comparison of testsfor specificity

Specificity of the 7 different serologic tests (WR, KT, VDRL, RPCF, TPI, FTA-200, RFTA-200) on the sera collected mainly from three sources where the incidence of biologically false positive reactors are more common.

They were:

1. Blood donors - Blood transfusion service, Royal Infirmary, Edinburgh.
2. Rheumatoid arthritis - Rheumatic Disease Unit, Northern General Hospital, Edinburgh.
3. Miscellaneous cases like pregnancy, tuberculosis, systemic lupus erythematosus, infectious mononucleosis. Their sera were collected from different clinics in Edinburgh.

315 samples of sera were collected from the above sources and the different qualitative serologic tests were performed on these sera. Those sera which showed negative results with the TPI tests but positive result with any of the non-specific tests like WR, KT, VDRL, were considered as biologically false positive sera. Fifty-seven samples of sera showed shuch reactive results out of 315 samples collected. Their reactivities were compared qualitatively with RPCF, FTA-200 and RFTA-200 tests.

The FTA test was also perfromed with these samples at 1:25, 1:50 and 1:100 dilutions to find out whether the reduction of serum dilution from the level used in the FTA-200 test has got any effect on the reactivity with biologically false positive sera.

Chapter IV

RESULTS

Comparison of tests for sensitivity

1. In experimental rabbit syphilis:

(a) Treated Group (5 rabbits) - The antibody titres observed with the seven serologic tests (WR, KT, VDRL, TPI, FTA, RPCF and RFTA) in the sera from each rabbit of this group during the period of 48 weeks are shown in separate tables along with their code numbers (Table III - B1, Table IV - B2, Table V - B4, Table VI - B5, Table VII - B6). The pattern of antibody titres are also shown graphically in figures (Fig. 2 - B1, Fig. 3 - B2, Fig. 4 - B4, Fig. 5 - B5, Fig. 6 - B6). The composite average titres are tabulated in Table XII and are shown graphically in Fig. 11, after drawing on a semilogarithm graph paper.

It is evident from Table XII and Fig. 11 that all the rabbits in this group showed non-specific serologic reactivities in their control sera with KT, FTA, RPCF and RFTA tests at different titres before inoculation with treponemes.

In the earlier part of the disease there is a sharp rise of antibody titre detected by the non-treponemal tests (WR, KT, VDRL) within 2 weeks of infection. RPCF and RFTA tests also showed a slight rise from their basic titres at this period. The TPI test manifested a late reactivity on the 4th week of infection whereas the FTA test showed a sudden rise of anti-

body titre within the first week of infection even surpassing the sensitivities of the non-treponemal group.

At the height of the infection all the tests behaved similarly in their sensitivities in reacting to a high titre including the FTA test which topped the list. A characteristic double peak in the antibody titre was noticed during this period with the non-treponemal group of tests.

After treatment the antibody titres of WR, KT, VDRL, RPCF and RFTA tests began to fall within a week and lowered to their basic titres at different intervals after completion of treatment (WR - 3 weeks, RPCF - 7 weeks, KT - 9 weeks, RFTA - 14 weeks, VDRL - 18 weeks). The TPI and FTA tests showed a slow fall in their titres during the whole post treatment period. The FTA test took a prolonged period of 26 weeks to become negative whereas the TPI test showed a positive reaction even on the 48th week though at a lower titre. So it is clear that the FTA test shows a sensitivity which runs midway between the TPI test and the remainder of the tests during the post treatment period.

(b) Untreated group (4 rabbits) - In this group the antibody titres of the sera are shown in separate tables for each rabbit along with their code numbers (Table VIII - B7, Table IX - B8, Table X - B9, Table XI - B10) and the pattern

of antibody titres are reproduced graphically in Fig. 7, 8, 9 and 10 respectively. The composite table (Table XIII) obtained from the average titres is shown graphically in Fig. 12 after drawing on a semilogarithm graph paper.

All the rabbits in this group showed non-specific reactivities in their control sera before infection with KT, RPCF, FTA and RFTA tests with the exception of rabbit No. B10 (Fig. 10) where no such reactivity was found with the RPCF test.

Table XIII and Fig. 12 show the relative sensitivities of the different tests at the later part of the disease in the untreated rabbits. It is clear that the TPI and the FTA tests showed a rise of titre in the earlier phase whereas all other tests showed a declining titre from the onset of the treatment. The WR became negative within 25 weeks of infection whereas KT and RFTA tests touched their base on the 38th week even without treatment. The VDRL test became negative in all the rabbits on the 48th week except in rabbit No. B9 (Fig. 9) which showed a weak reaction (Table X) at this stage. The RPCF test reacted at a poor titre till the 48th week of the disease but the TPI test maintained its reactivity throughout the period without any fall of its titre. Althouth the titre of the FTA test was lowered to a considerable

Table III Comparison of titres of different serologic tests at a period interval in Rabbit no. B1 inoculated intratesticularly with T. pallidum (Nichols strain). (Treated group).

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	±	-	5	10	+	-
1	-	±	-	10	200	+	-
2	160	32	64	20	200	5	-
3	80	16	32	20	200	10	-
4	80	16	32	10	400	20	-
5	80	32	64	20	400	20	+
6	80	32	64	40	400	40	3
7	160	32	64	320	800	40	3
8	80	16	32	160	800	40	9
9	40	8	32	80	800	40	9
10	40	8	32	80	1600	80	27
11	20	4	16	40	1600	80	81
12	20	4	16	40	1600	80	81
TREATMENT WITH PROCAINE PENICILLIN 30,000 UNITS DAILY FOR 8 DAYS							
14	5	2	8	20	800	80	81
15	-	2	8	20	800	40	81
16	-	2	4	20	800	40	81
17	-	2	4	10	800	40	81
18	-	2	4	10	800	20	81
19	-	2	4	10	400	20	81
20	-	+	2	10	400	20	81
21	-	+	2	5	200	20	81
22	-	+	2	5	200	10	27
23	-	±	2	5	200	10	27
24	-	±	2	5	200	10	27
25	-	±	+	5	200	5	27
26	-	±	+	5	200	5	27
27	-	±	±	5	200	5	27
28		±	±	5	200	+	27
30	-	±	-	5	200	+	9
32	-	±	-	5	100	+	9
34	-	±	-	5	100	+	9
36	-	±	-	5	100	+	9
38	-	±	-	5	100	+	9
40	-	±	-	5	10	+	3
44	-	±	-	5	10	+	3
48	-	±	-	5	10	+	3

Table IV Comparison of titres of different serologic tests at a periodic interval in Rabbit no.B_2 inoculated intratesticularly with T. pallidum (Nichols strain)

(Treated group)

Week after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	±	-	10	10	5	-
1	-	+	±	10	100	5	-
2	40	8	32	20	200	10	-
3	20	8	32	10	200	10	-
4	20	8	16	10	200	20	-
5	20	16	16	20	200	40	±
6	20	8	16	40	400	40	3
7	10	4	16	80	400	40	9
8	10	4	8	40	400	40	9
9	10	4	8	20	800	40	9
10	5	2	4	20	800	40	27
11	5	2	4	20	800	40	27
12	5	2	4	20	1600	40	81
TREATMENT WITH PROCAINE PENICILLIN 30,000 UNITS DAILY FOR 8 DAYS							
14	-	+	4	20	1600	40	81
15	-	+	2	20	800	20	81
16	-	+	2	20	800	20	81
17	-	+	2	20	800	20	81
18	-	+	2	10	800	20	81
19	-	+	2	10	400	20	81
20	-	+	2	10	400	20	81
21	-	+	2	10	200	10	27
22	-	±	+	10	200	10	27
23	-	±	+	10	200	10	27
24	-	±	±	10	200	10	27
25	-	±	±	10	200	10	27
26	-	±	±	10	200	5	27
27	-	±	±	10	200	5	27
28	-	±	-	10	200	5	27
30	-	±	-	10	200	5	9
32	-	±	-	10	100	5	9
34	-	±	-	10	100	5	9
36	-	±	-	10	100	5	9
38	-	±	-	10	10	5	3
40	-	±	-	10	10	5	3
44	-	±	-	10	10	5	3
48	-	±	-	10	10	5	+

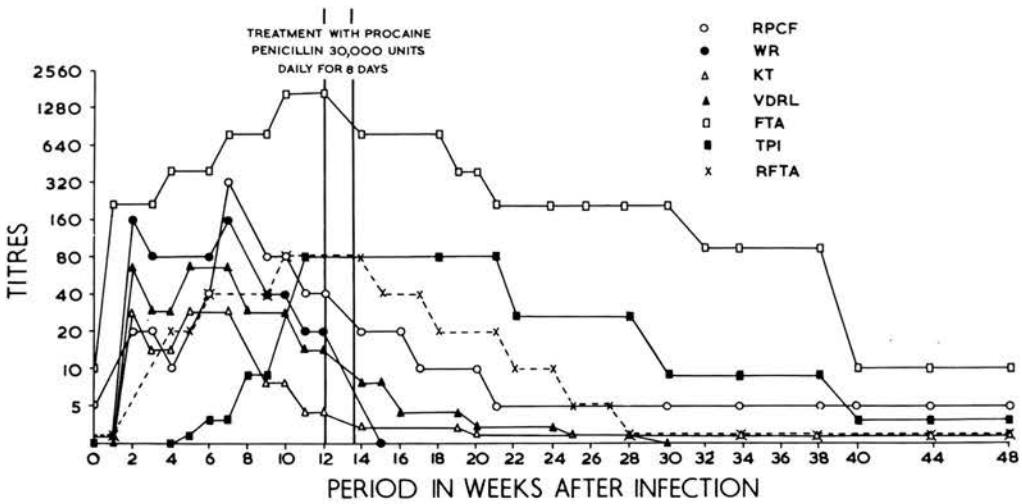

Fig. 2. Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B1 (treated group).

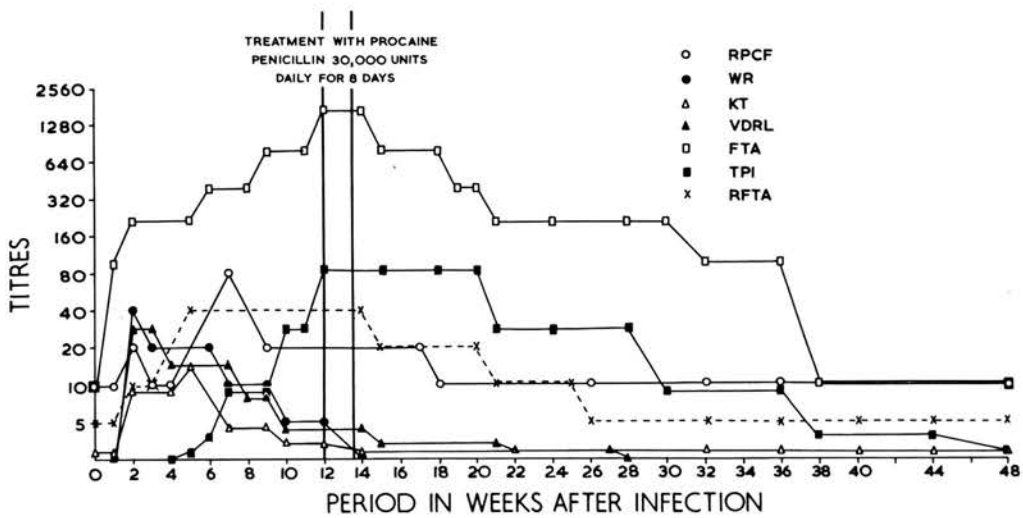

Fig. 3. Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B2 (treated group).

Table V Comparison of titres of different serologic tests at a periodic interval in Rabbit no. B_4 inoculated intratesticularly with T. pallidum (Nichols strain)

(Treated group)

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	±	-	10	10	+	-
1	-	2	±	20	100	+	-
2	40	8	16	20	200	5	-
3	80	16	32	20	200	5	-
4	40	64	64	20	200	10	3
5	40	16	32	40	200	20	9
6	40	8	32	320	400	40	9
7	40	16	32	80	400	40	27
8	40	8	16	40	400	40	81
9	20	8	16	40	800	40	81
10	20	8	16	40	800	40	81
11	20	4	8	40	1600	80	81
12	10	4	8	40	1600	80	81
TREATMENT WITH PROCAINE PENICILLIN 30,000 UNITS DAILY FOR 8 DAYS							
14	5	2	8	20	800	80	81
15	-	2	4	20	800	40	81
16	-	2	4	20	800	40	81
17	-	2	4	20	800	20	81
18	-	2	4	20	800	20	81
19	-	+	2	10	400	20	81
20	-	+	2	10	400	20	81
21	-	±	2	10	400	20	81
22	-	±	+	10	400	10	81
23	-	±	+	10	200	10	27
24	-	±	±	10	200	10	27
25	-	±	±	10	200	5	27
26	-	±	±	10	200	5	27
27	-	±	±	10	200	5	27
28	-	±	±	10	200	+	27
30	-	±	-	10	200	+	9
32	-	±	-	10	100	+	9
34	-	±	-	10	100	+	9
36	-	±	-	10	100	+	9
38	-	±	-	10	10	+	3
40	-	±	-	10	10	+	3
44	-	±	-	10	10	+	3
48	-	±	-	10	10	+	+

Table VI Comparison of titres of different serologic tests at a periodic interval in Rabbit no. B_5 inoculated intra-testicularly with T. pallidum (Nichols strain)

(Treated group)

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	±	-	10	10	5	-
1	-	+	±	20	100	5	-
2	40	16	16	20	200	10	-
3	160	64	64	20	200	10	-
4	80	32	32	20	200	20	3
5	80	32	64	40	400	20	9
6	80	16	32	320	400	40	9
7	80	16	32	80	400	40	9
8	80	16	32	40	400	40	9
9	80	16	32	40	800	40	27
10	40	16	32	40	800	40	27
11	40	16	32	40	1600	80	81
12	40	8	16	40	1600	80	81
TREATMENT WITH PROCAINE PENICILLIN 30,000 UNITS DAILY FOR 8 DAYS							
14	5	4	16	20	800	80	81
15	5	4	8	20	800	80	81
16	5	4	8	20	800	40	81
17	-	2	4	20	800	40	81
18	-	2	4	20	800	20	81
19	-	2	4	20	400	20	81
20	-	2	4	10	400	20	81
21	-	2	4	10	200	20	81
22	-	2	4	10	200	10	27
23	-	+	2	10	200	10	27
24	-	+	2	10	200	10	27
25	-	±	+	10	200	10	27
26	-	±	+	10	200	10	27
27	-	±	+	10	200	5	27
28	-	±	±	10	200	5	27
30	-	±	±	10	200	5	9
32	-	±	-	10	100	5	9
34	-	±	-	10	100	5	9
36	-	±	-	10	100	5	9
38	-	±	-	10	10	5	9
40	-	±	-	10	10	5	3
44	-	±	-	10	10	5	3
48	-	±	-	10	10	5	3

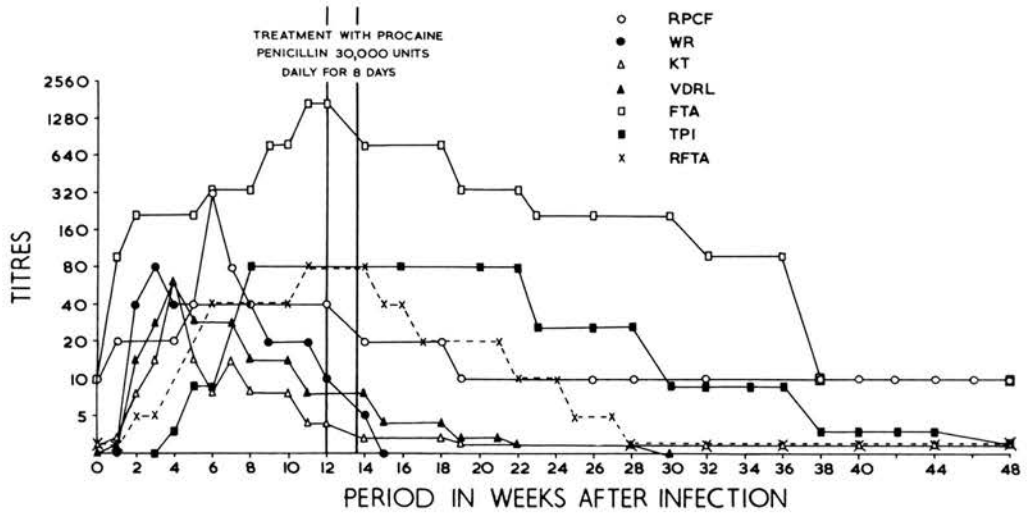

Fig. 4. Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B4 (treated group).

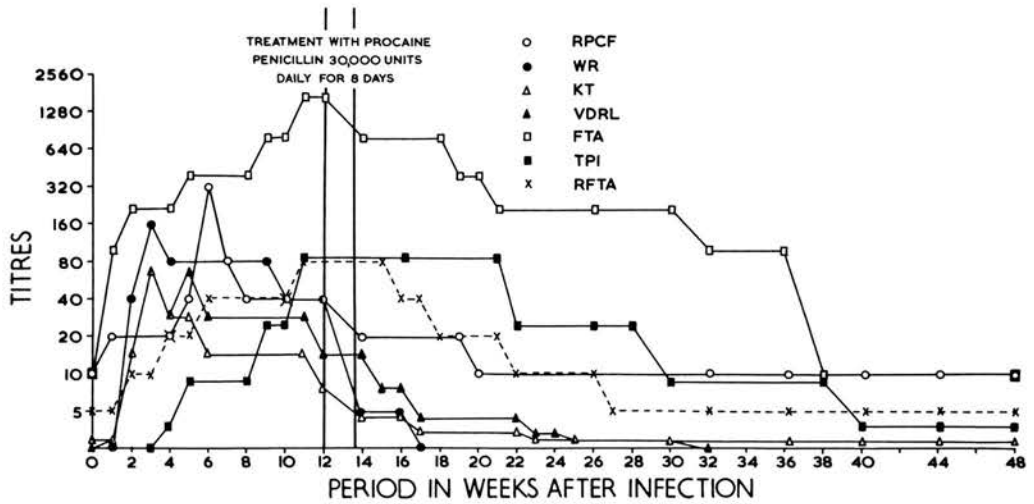

Fig. 5. Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B5 (treated group).

Table VII Comparison of titres of different serologic tests at a periodic interval in Rabbit no. B6 inoculated intra-testicularly with T. pallidum (Nichols strain)

(Treated group)

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	±	-	10	10	+	-
1	-	2	2	10	100	+	-
2	80	16	16	10	200	5	-
3	80	32	64	10	200	10	-
4	40	32	32	10	200	20	+
5	40	16	32	20	400	40	3
6	40	16	32	80	400	40	9
7	40	16	64	40	400	40	9
8	80	32	64	80	800	40	9
9	80	32	64	80	800	40	9
10	80	32	64	80	1600	40	27
11	80	16	32	80	1600	80	27
12	40	16	32	80	1600	80	81
TREATMENT WITH PROCAINE PENICILLIN 30,000 UNITS DAILY FOR 8 DAYS							
14	10	8	32	40	800	80	81
15	5	4	8	20	800	80	81
16	5	4	8	20	800	40	81
17	-	2	4	20	800	40	81
18	-	2	4	20	800	40	81
19	-	2	4	20	800	20	81
20	-	2	4	10	400	20	81
21	-	2	4	10	400	20	81
22	-	2	2	10	200	20	27
23	-	+	2	10	200	10	27
24	-	+	2	10	200	10	27
25	-	±	+	10	200	10	27
26	-	±	+	10	200	5	27
27	-	±	+	10	200	5	27
28	-	±	±	10	200	+	27
30	-	±	±	10	200	+	9
32	-	±	-	10	100	+	9
34	-	±	-	10	100	+	9
36	-	±	-	10	100	+	9
38	-	±	-	10	100	+	9
40	-	±	-	10	10	+	3
44	-	±	-	10	10	+	3
48	-	±	-	10	10	+	3

Table VIII Comparison of titres of different serologic tests at a periodic interval in Rabbit no. B_7 inoculated intra-testicularly with *T. pallidum* (Nichols strain)

(Untreated group)

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	±	-	5	10	+	-
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14	10	4	8	20	1600	80	81
15	10	4	8	20	1600	80	81
16	5	4	8	20	1600	80	81
17	-	4	8	20	1600	80	243
18	-	2	4	20	3200	80	243
19	-	2	4	20	3200	80	243
20	-	2	4	20	1600	80	243
21	-	2	4	20	1600	80	243
22	-	2	4	20	800	80	243
23	-	2	4	20	800	40	243
24	-	2	4	20	800	40	243
25	-	2	4	20	800	40	243
26	-	2	4	20	800	40	243
27	-	2	2	20	800	20	243
28	-	+	2	10	400	20	243
30	-	+	2	10	400	20	243
32	-	+	2	10	400	10	243
34	-	+	2	10	200	5	243
36	-	+	2	10	200	5	243
38	-	+	2	10	200	+	243
40	-	±	2	10	200	+	243
44	-	±	±	10	200	+	243
48	-	±	-	10	200	+	243

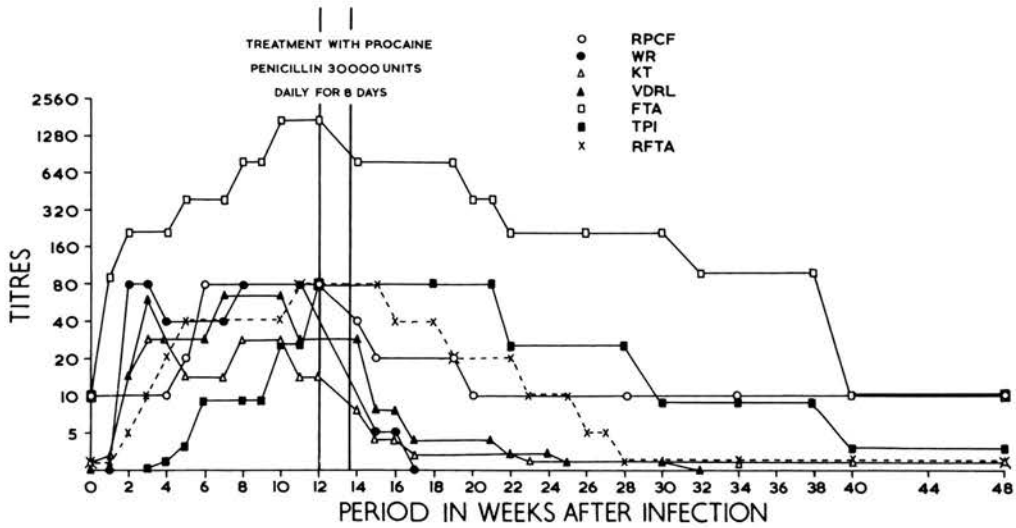

Fig. 6. Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B6 (treated group).

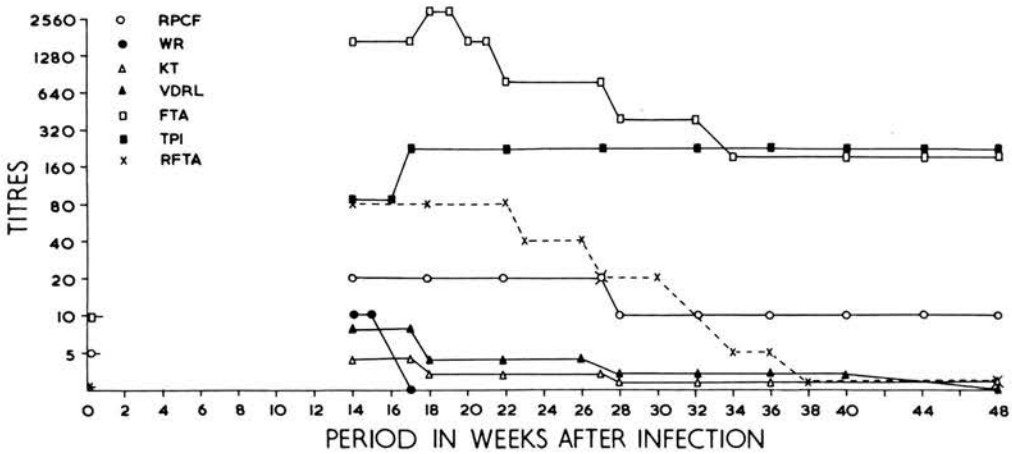

Fig. 7 Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B7 (untreated group).

Table IX Comparison of titres of different serologic tests at a periodic interval in Rabbit no. B_8 inoculated intra-testicularly with T. pallidum (Nichols strain)

(Untreated Group)

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	±	-	5	10	5	-
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14	5	4	8	20	1600	40	81
15	5	4	8	20	1600	40	81
16	-	4	8	20	1600	40	81
17	-	2	4	20	1600	40	81
18	-	2	4	20	1600	40	243
19	-	2	4	20	1600	40	243
20	-	2	4	20	1600	40	243
21	-	2	4	20	800	40	243
22	-	2	4	20	800	40	243
23	-	2	4	20	800	20	243
24	-	2	4	20	800	20	243
25	-	2	4	20	800	20	243
26	-	2	4	20	800	20	243
27	-	2	4	10	400	10	243
28	-	+	2	10	400	10	243
30	-	+	2	10	400	10	243
32	-	+	2	10	400	10	243
34	-	+	2	10	200	10	243
36	-	+	2	10	200	5	243
38	-	+	2	10	200	5	243
40	-	+	2	10	200	5	243
44	-	±	±	10	200	5	243
48	-	±	-	10	200	5	243

Table X Comparison of titres of different serologic tests at a periodic interval in Rabbit no. B_9 inoculated intra-testicularly with T. pallidum (Nichols strain)

(Untreated group)

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	±	-	5	10	5	-
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14	10	8	16	20	1600	80	81
15	10	4	8	20	1600	80	81
16	10	4	8	20	1600	80	81
17	5	4	8	20	1600	80	81
18	5	4	8	20	3200	80	243
19	5	4	8	20	3200	40	243
20	5	2	4	20	1600	40	243
21	5	2	4	20	1600	40	243
22	5	2	4	20	800	40	243
23	±	2	4	20	800	40	243
24	±	2	4	20	800	40	243
25	-	2	4	20	800	40	243
26	-	2	4	20	800	40	243
27	-	2	4	20	800	40	243
28	-	2	4	10	400	40	243
30	-	2	4	10	400	20	243
32	-	2	4	10	400	20	243
34	-	2	4	10	400	10	243
36	-	2	4	10	200	10	243
38	-	+	4	10	200	5	243
40	-	±	2	10	200	5	243
44	-	±	+	10	200	5	243
48	-	±	±	10	200	5	243

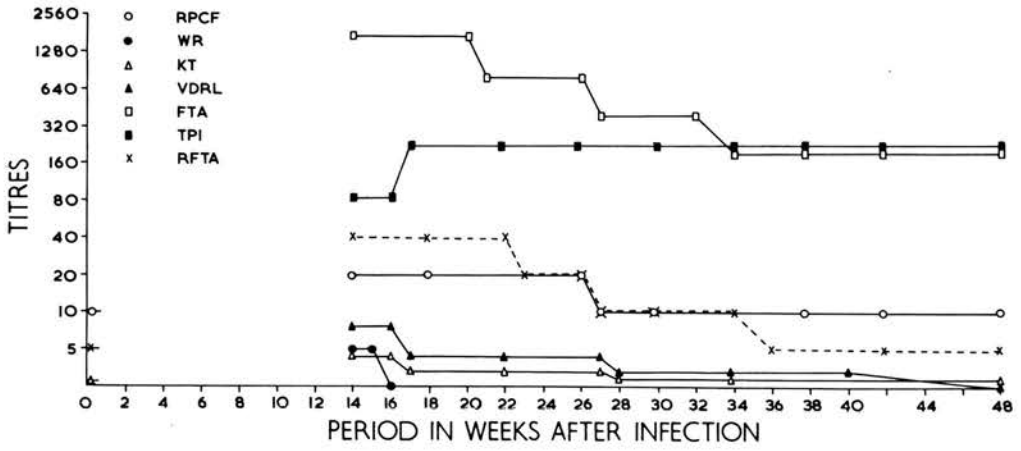

Fig. 8. Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B8 (untreated group).

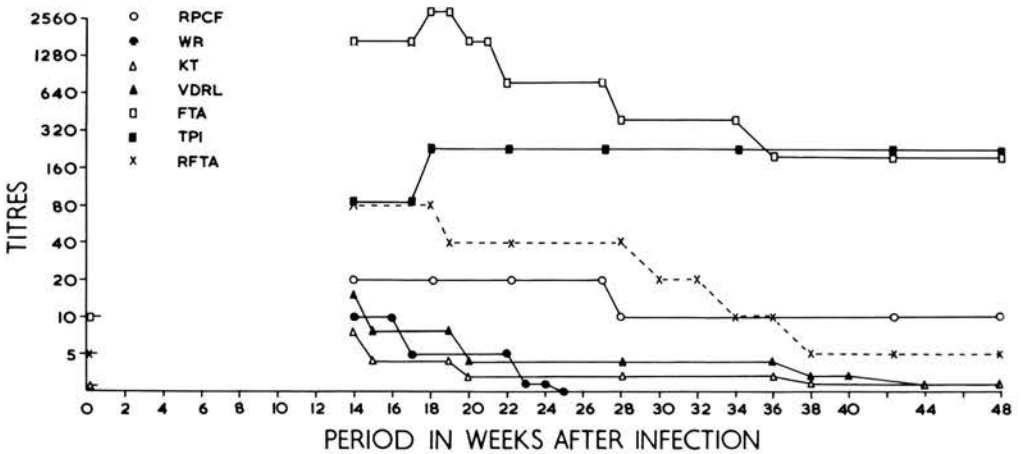

Fig. 9. Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B9 (untreated group).

Table : XI Comparison of titres of different serologic tests at a periodic interval in Rabbit No. B10 inoculated intratesticularly with T. pallidum (Nichols strain). (Untreated group).

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	+	-	-	5	+	-
1		-					
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14	10	8	16	40	1600	80	81
15	5	8	16	20	1600	80	81
16	5	4	8	20	1600	80	81
17	5	4	8	20	1600	80	81
18	5	4	8	20	1600	40	243
19	5	2	4	20	1600	40	243
20	5	2	4	10	800	40	243
21	5	2	4	10	800	40	243
22	-	2	4	10	800	40	243
23	-	2	4	10	800	40	243
24	-	2	4	10	800	40	243
25	-	2	4	10	800	40	243
26	-	2	4	5	400	40	243
27	-	2	4	5	400	20	243
28	-	+	2	5	400	20	243
30	-	+	2	5	400	20	243
32	-	+	2	5	400	10	243
34	-	+	2	5	200	5	243
36	-	+	2	5	200	5	243
38	-	+	2	5	200	5	243
40	-	±	2	5	200	+	243
44	-	±	±	5	200	+	243
48	-	-	-	5	200	+	243

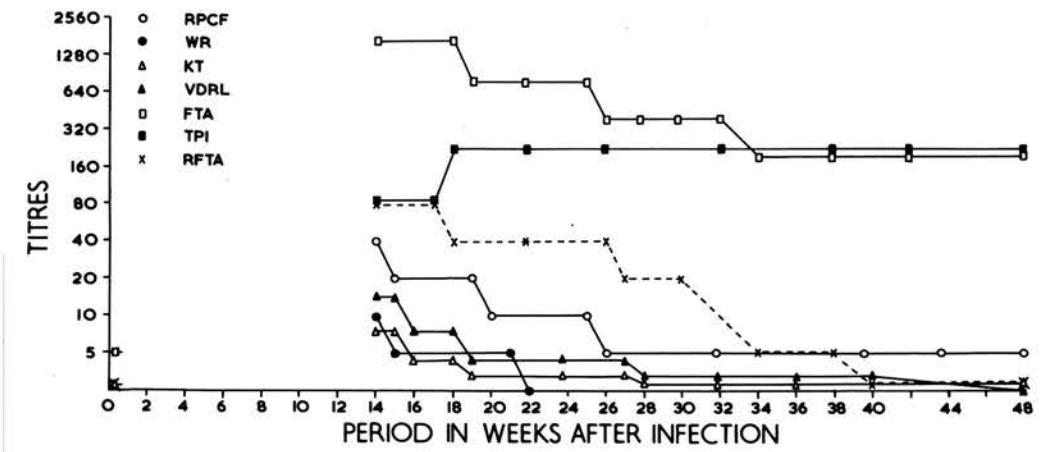

Fig. 10. Antibody patterns following intratesticular inoculation of T. pallidum in Rabbit No. B10 (untreated group).

Table XII Composite table showing titres of different serologic tests at a periodic interval in treated group of rabbits inoculated intratesticularly with T. pallidum.

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	1	-	9	10	2.6	-
1	-	1.4	1	14	120	3.6	-
2	92	16	28.8	18	200	8	-
3	84	27.2	44.8	16	200	9	-
4	52	29.6	35.2	14	240	18	1.6
5	52	22.4	41.2	28	320	28	5
6	52	16	23.2	160	400	40	6
7	66	19.2	41.6	110	480	40	11.4
8	58	15.2	30.4	72	560	40	23.4
9	46	13.6	30.4	52	800	40	30.6
10	37	12.8	29.6	52	1120	49	37.8
11	33	8.4	18.4	44	1440	72	70.2
12	23	6.8	15.2	44	1600	72	81
TREATMENT WITH PROCAINE PENICILLIN 30,000 UNITS DAILY FOR 8 DAYS							
14	5	4.2	13.6	24	960	72	81
15	2	2.6	6	20	800	52	81
16	2	2.2	5.2	20	800	36	81
17	-	1.8	3.6	18	800	32	81
18	-	1.8	3.6	16	800	24	81
19	-	1.6	3.2	14	480	20	81
20	-	1.4	3.2	10	400	20	81
21	-	1.4	2.8	9	280	18	70.2
22	-	1.2	2.0	9	240	12	37.8
23	-	1	1.6	9	200	10	27
24	-	1	1.6	9	200	10	27
25	-	1	1.0	9	200	8	27
26	-	1	1.0	9	200	6	27
27	-	1	1.0	9	200	5	27
28	-	1	.8	9	200	2.6	27
30	-	1	.4	9	200	2.6	9
32	-	1	-	9	100	2.6	9
34	-	1	-	9	100	2.6	9
36	-	1	-	9	100	2.6	9
38	-	1	-	9	46	2.6	6.6
40	-	1	-	9	10	2.6	3
44	-	1	-	9	10	2.6	3
48	-	1	-	9	10	2.6	2.2

Table XIII Composite table showing the titres of different serologic tests at a periodic interval in untreated group of rabbits inoculated intratesticularly with T. pallidum.

Weeks after infection	Serologic tests						
	WR (Price's)	KT	VDRL	RPCF	FTA	RFTA	TPI
0	-	1	-	3.75	8.75	3	-
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14	8.75	6	12	25	1600	70	81
15	7.5	5	10	25	1600	70	81
16	5	4	8	25	1600	70	81
17	2.5	3.5	7	25	1600	70	121.5
18	2.5	3	6	25	2400	60	243
19	2.5	2.5	5	25	2400	50	243
20	2.5	2	4	17.5	1400	50	243
21	2.5	2	4	17.5	1200	50	243
22	1.25	2	4	17.5	800	50	243
23	.25	2	4	17.5	800	35	243
24	.25	2	4	17.5	800	35	243
25	-	2	4	17.5	800	35	243
26	-	2	4.	16.25	700	35	243
27	-	2	3.5	13.75	600	22.5	243
28	-	1.25	2.5	8.75	400	22.5	243
30	-	1.25	2.5	8.75	400	17.5	243
32	-	1.25	2.5	8.75	400	12.5	243
34	-	1.25	2.5	8.75	250	7.5	243
36	-	1.25	2.5	8.75	200	6.25	243
38	-	1	2	8.75	200	3	243
40	-	1	2	8.75	200	3	243
44	-	1	1	8.75	200	3	243
48	-	1	.25	8.75	200	3	243

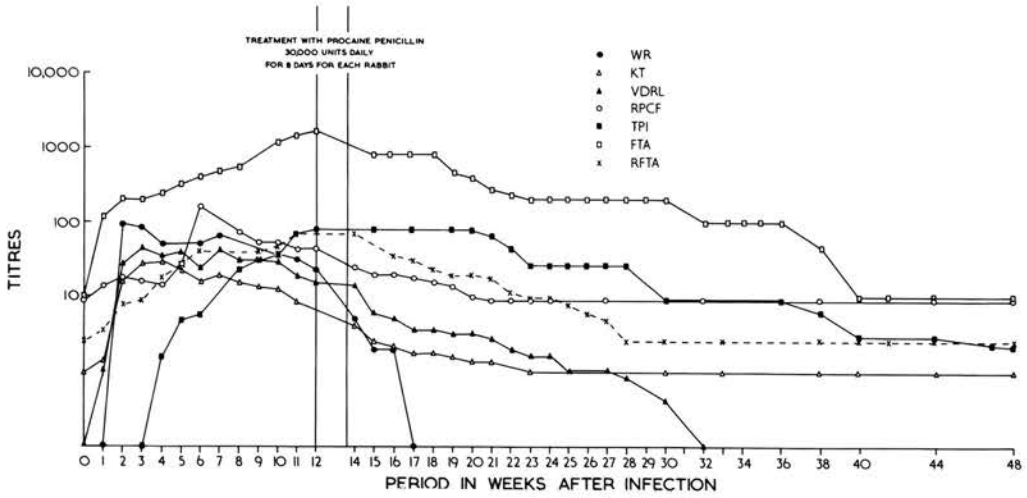

Fig. 11. Composite chart showing the titre of different serologic tests at a periodic interval in the treated group of rabbits after intratesticular inoculation of T. pallidum.

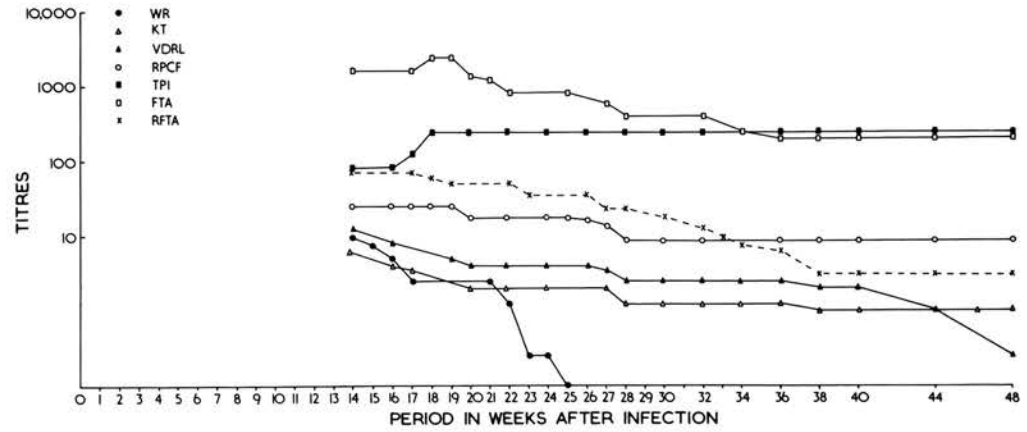

Fig. 12. Composite chart showing the titre of different serologic tests at a periodic interval in the untreated group of rabbits after intratesticular inoculation of T. pallidum.

extent during this period, it still showed reactivity at a much higher dilution in comparison with its basic titre and could compete with the TPI in relation to its sensitivity even at this late stage of the disease.

2. In early primary human syphilis:

The reactivities of the seven serologic tests (WR, KT, VDRL, TPI, FTA-200, RPCF, RFTA-200) carried out qualitatively on the sera from 25 cases of early primary syphilis are compared in Table XIV. In this group the non-treponemal tests (WR, KT, VDRL) are sensitive enough to find out 16 cases out of the total 25 sera. The RPCF behaved similarly with the non-treponemal group in its sensitivity. The TPI test showed a poor sensitivity by finding out 11 cases out of the total 25 sera. The FTA-200 test compared favourably with all the non-treponemal tests in the diagnosis of these early cases and even showed more sensitive results than the latter in finding out two more cases (serum No. FT9 and FT16) from this group. In comparison with the FTA-200 test, RFTA-200 test showed poor sensitivity as it could not diagnose even a single case from this group at 1:200 dilution of the sera.

The results of the quantitative FTA test performed on the non-reactive FTA-200 sera from this group of early primary

Table XIV Comparative reactivities of the different serologic tests with the sera from cases of early primary human syphilis.

sr. no.	serum no.	Serologic tests						
		WR (Pr.)	KT	VDRL	RPCF	FTA-200	RFTA-200	TPI
1	FTI	±	+	±	+	++	-	±
2	FT2	-	-	-	-	-	-	-
3	FT3	±	+	±	±	++	-	-
4	FT4	+	+++	++	++	+++	-	+
5	FT5	+	++	±	±	++	-	-
6	FT6	++	++	++	++	+++	-	±
7	FT7	+	++	++	+	++	-	-
8	FT8	±	++	++	+	++	-	-
9	FT9	-	-	-	-	++	-	-
10	FT10	-	-	-	-	-	-	-
11	FT11	-	-	-	-	-	-	-
12	FT12	++	+++	++	++	+++	-	+
13	FT13	+	++	++	+	++	-	+
14	FT14	+	++	+	±	++	-	+
15	FT15	-	-	-	-	-	-	-
16	FT16	-	-	-	-	++	-	-
17	FT17	++	++	++	+	+++	-	+
18	FT18	-	-	-	-	-	-	-
19	FT19	++	++	++	++	+++	-	±
20	FT20	-	-	-	-	-	-	-
21	FT21	-	-	-	-	-	-	-
22	FT24	++	+++	++	++	+++	-	±
23	FT25	+	++	++	+	+++	-	+
24	FT26	±	+	+	±	++	-	-
25	FT27	+	++	++	+	+++	-	+

Table XV shows the titres of quantitative FTA tests with the normal sera in comparison with the titres of non-reactive FTA-200 sera from early syphilis.

Normal sera					Normal sera					Non-reactive FTA-200 early Syph. sera.				
Serum no.	Titre of the FTA test 1:5	1:10	1:25	1:50	Serum no.	Titre of the FTA test 1:5	1:10	1:25	1:50	Serum no.	Titre of the FTA test 1:10	1:25	1:50	1:100
BT1	+	±	-		BT14	±	-			FT2	++	++	±	-
BT2	+	-			BT15	-				FT10	++	++	+	±
BT3	+	-			BT16	-				FT12	++	++	+	±
BT4	++	+	-		BT17	±	-			FT15	++	++	+	±
BT5	±	-			BT18	-				FT18	++	++	+	-
BT6	-				BT19	++	±	-		FT20	+++	++	++	++
BT7	-				BT20	±	-			FT21	++	++	±	-
BT8	+	-			BT21	-								
BT9	-				BT22	-								
BT10	-				BT23	-								
BT11	-				BT24	±	-							
BT12	+	-			BT25	-								
BT13	±	-												

cases are compared in Table XV along with the results of similar tests carried out on 25 samples of normal sera collected from the healthy blood donors which showed negative results with the other six serologic tests. The results show an upper level of reactivity at 1:10 dilution in 3 sera out of 25 samples in the latter group whereas non-reactive FTA-200 sera showed a positive reaction at 1:25 dilution in all the sera except in one (serum No. FT20) where such reactivity was found to be at 1:100.

Comparison of tests for specificity:

The comparative reactivity of the seven serologic tests (WR, KT, VDRL, TPI, FTA-200, RPCF, RFTA-200) with the 57 biologically false positive sera surveyed in this study are given in three separate tables (Tables XVI, XVII, XVIII). This division was based mainly on the source of these sera. Among the 57 sera included, 23 samples were from normal healthy blood donors after repeated donation, 25 samples from cases of rheumatoid arthritis and the rest was included from a miscellaneous group consisting of nephrotic syndrome, pulmonary tuberculosis, glandular fever, systemic lupus erythematosus, pregnancy and erythema nodosum.

Table XVI showing serologic reactions of "biologically false positive" sera.

Group A. Normal healthy blood donors (after repeated donation)								
Sr. no.	Serum no.	Serologic tests						
		WR (Pr.)	KT	VDRL	RPCF	FTA: 200	RFTA: 200	TPI
1	8	-	±	-	-	-	-	-
2	15	±	±	±	-	-	-	-
3	17	±	±	±	-	-	-	-
4	18	+	±	±	±	-	-	-
5	21	+	±	+	+	-	-	-
6	22	-	±	±	-	-	-	-
7	24	-	±	±	-	-	-	-
8	41	+	+	+	-	-	-	-
9	55	-	±	±	-	-	-	-
10	89	-	+	±	-	-	-	-
11	103	-	±	-	-	-	-	-
12	104	-	+	+	-	-	-	-
13	106	-	±	-	-	-	-	-
14	110	-	±	-	-	-	-	-
15	111	-	+	-	-	-	-	-
16	112	-	±	-	-	-	-	-
17	116	+	+	-	-	-	-	-
18	118	-	+	-	-	-	-	-
19	120	-	±	+	-	-	-	-
20	121	-	±	±	-	-	-	-
21	115	+	+	+	-	-	-	-
22	262	-	+	+	-	-	-	-
23	264	+	+	+	-	-	-	-

Table XVII showing serologic reactions of "biologically false positive" sera.

Group B. Rheumatoid arthritis.								
Sr. no.	Serum no.	Serologic tests						
		WR (Pr.)	KT	VDRL	RPCF	FTA: 200	RFTA: 200	TPI
1	27	-	±	-	±	-	-	-
2	31	-	+	±	±	-	-	-
3	35	±	+	±	±	-	-	-
4	71	-	+	-	-	-	-	-
5	72	-	+	-	-	-	-	-
6	93	-	+	±	-	-	-	-
7	94	-	+	-	-	-	-	-
8	144	+	+	+	-	-	-	-
9	162	-	+	±	-	-	-	-
10	166	-	+	-	-	-	-	-
11	168	-	+	-	-	-	-	-
12	176	-	+	-	-	-	-	-
13	181	-	+	+	-	-	-	-
14	190	-	±	-	-	-	-	-
15	195	-	±	-	-	-	-	-
16	202	-	+	+	-	-	-	-
17	211	-	±	-	-	-	-	-
18	213	-	+	-	-	-	-	-
19	216	-	±	-	-	-	-	-
20	224	-	+	+	-	-	-	-
21	235	-	+	-	-	-	-	-
22	248	-	+	±	-	-	-	-
23	250	-	±	-	-	-	-	-
24	255	-	+	-	-	-	-	-
25	267	-	+	±	-	-	-	-

Table XVIII showing serologic reactions of "biologically false positive" sera.

Group C. Miscellaneous									
			Serologic tests						
Sr. no.	serum no.	cl. diagnosis	WR (Pr.)	KT	VDRL	RPCF	FTA: 200	RFTA: 200	TPI
1	3	Nephrotic syndrome	±	±	±	-	-	-	-
2	45	Chronic vulval ulcer	±	±	-	-	-	-	-
3	79	Pulm. Tubercul-osis	+	+	+	-	-	-	-
4	100	Ischaemic arterial disease	-	±	+	-	-	-	-
5	101	Glandular fever	-	±	-	-	-	-	-
6	109	S. L. E.	-	+	+	-	-	-	-
7	124	Pregnancy	+	+	+	-	-	-	-
8	131	Erythema nodosum	+	+	-	-	-	-	-
9	155	Pregnancy	-	±	+	+	-	-	-

Table XIX Comparative specificity of seven serologic tests in biologically false positive sera.

Tests	No. of sera tested	Negative	Specificity (%)
TPI	57	57	100
FTA-200	57	57	100
RFTA-200	57	57	100
RPCF	57	5	91.3
WR (Price's)	57	12	79
VDRL	57	31	45.5
KT	57	Nil	0.0

Table XIX shows the comparative specificity of the seven serologic tests in relation to biologically false positive sera. As the specificity of the tests was judged mainly from the reactivity of the TPI test, considering it as a specific standard test, it showed 100% specificity in comparison with KT, which was reactive with all the sera of this group. FTA-200 and RFTA-200 tests compared favourably with the TPI test showing 100% specificity whereas the RPCF, WR and VDRL showed 91.3%, 79% and 45.5% respectively.

The specificity of the FTA test was judged again in this

Table XX Shows the titres of quantitative FTA tests with the sera from biologically false positive reactors.

Group A Normal healthy blood donors					Group B Rheumatoid arthritis					Group C Miscellaneous				
Sr. no.	Serum no.	Titre of FTA test 1:25	1.50	1:100	Sr. no.	Serum no.	Titre of FTA test 1:25	1:50	1:100	Sr. no.	Serum no.	Titre of FTA test 1:25	1:50	1:100
1	8	+	±	-	1	27	-			1	3	±	-	
2	15	++	±	-	2	31	-			2	45	-		
3	17	-			3	35	±	-		3	79	-		
4	18	±	-		4	71	-			4	100	±	-	
5	21	+	±	-	5	72	++	++	±	5	101	±	-	
6	22	-			6	93	-			6	109	±	-	
7	24	±	-		7	94	-			7	124	+	-	
8	41	-			8	144	++	+	-	8	131	±	-	
9	55	++	++	+	9	162	-			9	155	±	-	
10	89	±	-		10	166	±	-						
11	103	+	-		11	168	++	+	-					
12	104	-			12	176	+	-						
13	106	-			13	181	-							
14	110	±	-		14	190	-							
15	111	+	-		15	195	-							
16	112	++	±	-	16	202	-							
17	116	±	-		17	211	-							
18	118	-			18	213	±	-						
19	120	-			19	216	±	-						
20	121	-			20	224	-							
21	115	++	±	-	21	235	-							
22	262	++	+	-	22	248	-							
23	264	-			23	250	±	-						
					24	255	±	-						
					25	267	+	±	-					

group of 57 sera at 1:25, 1:50 and 1:100 dilutions as shown in Table XX. It is clear from the table that 2 sera showed non-specific reactivity when the test was performed at 1:100 dilution, 8 more sera showed such reactivity at 1:50 dilution and 22 sera became reactive at 1:25 dilution.

Reproducibility of the tests

Using the positive sera of known titre as controls in each series of tests the results were found to be reproducible within one tube (doubling dilution) in all the tests used in this investigation.

To sum up the results, it is found that the non-treponemal, the TPI and the newer treponemal tests behaved in their sensitivity and specificity in the following way:

Non-treponemal tests (WR - Price's, KT, VDRL): All the tests behaved similarly in their sensitivity and showed a more sensitive reaction than the TPI in the early stage of human infection. When the early stage of rabbit syphilis is studied, the WR behaved like the least sensitive test in this group as KT and VDRL tests showed an earlier rise in their titre in the first week of infection in comparison with the WR, which became positive in the second week of infection.

Although in the well-developed stage of the disease in the rabbit all the tests reacted at a high titre, KT showed the least —

sensitive result as its titre was lower than the WR and VDRL tests at this stage of infection.

In the treated late stage of rabbit syphilis, the VDRL, KT and WR showed their positions in terms of sensitivity in that order as the VDRL test took 18 weeks to become negative and the WR showed negative result on the 3rd week after treatment. In late untreated cases of rabbit's infection, the tests behaved similarly to the treated group in their sensitivity but the VDRL test remained positive for a long time, i.e. until the end (48th week) of the observation, whereas the WR became negative on the 38th week of infection.

When the specificity of these tests is studied in biologically false positive reactors, WR (Price's) showed the best result as it gave a specificity of 79% in comparison with the VDRL (45.5%) and KT (0.0%). The result of poor specificity of KT was also supported by its non-specific reactions with all the rabbit sera before infection where the WR and VDRL tests showed negative results.

TPI test: In the earlier stage of both rabbit syphilis and human infection, the TPI test showed less sensitive reactions than the non-treponemal group of tests as this TPI test took a long time after infection to manifest a positive result. In the advanced stage of the disease in rabbits, the test showed a

sensitivity similar to that of the non-treponemal group of tests as the titre was high enough to compete with these tests. In the late stage of experimental rabbit syphilis, the TPI test gave more sensitive results than the non-treponemal tests in both treated and untreated groups in showing positive results when all the non-treponemal tests were negative (except the VDRL test in the untreated late stage of the disease).

The TPI test also showed a 100% specificity when biologically false positive sera were studied as the test was considered as the standard test of specificity for syphilis.

Newer treponemal tests:

RPCF: Although this test showed a more sensitive result than the TPI in both early stages of rabbit and human infection and behaved similarly to the KT and VDRL in the former group, it reacted like the Wassermann reaction (Price's) in the latter group along with the KT and VDRL tests. In the florid stage of the disease in the rabbit, the RPCF test showed results similar to the TPI and non-treponemal group of tests. In the late stage of infection in untreated rabbit the test remained positive till the 48th week of infection and showed a sensitivity similar to the TPI and VDRL tests in manifesting a positive result at this stage although the titre was much lower than the TPI test. But in the treated late stage of rabbit syphilis, the

RPCF test became negative earlier and showed sensitivity similar to the KT of the non-treponemal group as the RPCF test became negative after 7 weeks of treatment.

In biologically false positive sera the RPCF test showed a specificity of 91.3% which is much better than the non-treponemal group but in the rabbit sera before infection, this test showed a non-specific reaction in a fair number of sera where WR and VDRL tests showed negative results.

RFTA: The RFTA test showed a sensitive result like the KT and VDRL tests of the non-treponemal group in the early stage of rabbit syphilis. In the advanced stage of the disease in rabbit, the titre was comparative in sensitivity to all other tests and even showed a higher titre than the KT. In the untreated stage of infection in rabbit, the test manifested a similar sensitivity to the KT in showing its basic titre on the 38th week of infection whereas in the treated group it showed less sensitive results than the VDRL test. The RFTA-200 test showed the least sensitive results amongst all the tests in the early stages of human infection as the test did not react with any sera from this group when all the other tests showed positive results.

The specificity of the RFTA-200 test was found to be similar to the FTA-200 and TPI tests in biologically false posi-

tive sera although the RFTA test showed non-specific reactions with all the rabbit sera before infection.

FTA: The FTA test showed the most sensitive result in the early and advanced stages of rabbit syphilis in comparison with the WR, KT, VDRL tests of the non-treponemal group, the TPI test and the RPCF test of the newer treponemal group. In the late stage of untreated rabbit syphilis, the test remained positive at a high dilution like the TPI test on the 48th week of infection and showed more sensitive results than the VDRL and RPCF tests which also showed positive results at this stage. In the treated late stage of experimental rabbit infection, the FTA test came down to its basic titre after a prolonged period of 26 weeks following treatment whereas the non-treponemal group and RPCF tests became negative earlier after 18 weeks of treatment and the TPI test still showed a positive result at this stage.

In the early stage of human syphilis although the FTA-200 test showed most sensitive results amongst all the serologic tests used for this study, it still failed to react with all the sera in this group.

When the specificity of the FTA-200 test was judged in biologically false positive sera, the test showed an absolute specificity of 100% like the TPI test but the FTA test showed non-specific reactions at low dilution (1:10) with most of the

rabbit sera before infection.

Attempts have been made to increase the sensitivity of the FTA-200 test by lowering the testing dilution of serum from 1:200 to 1:25 to see whether such an FTA-25 test will react with all the sera from early cases of syphilis. The results of the FTA-25 test showed reactivity with all the sera from early cases but at this dilution most of the sera from the biologically false positive group showed positive results although no such reactivity was found with the normal sera in such dilution.

Chapter V
COMPARATIVE ANALYSIS OF THE SENSITIVITY AND SPECIFICITY OF TREPONEMAL AND NON-TREPONEMAL TESTS

The result of an evaluation study related to any diagnostic laboratory procedure is assessed mainly from its relationship with the clinical disease so that the diagnosis, prognosis and treatment of the disease concerned could be guided by the sensitivity of the test if the test is found to be suitable. At the same time the reactivity of the test should be restricted only to the particular disease if the test is to be declared as specific.

Evaluation of sensitivity

In this study the sensitivities of the newer treponemal tests (RPCF, RFTA and FTA) have been evaluated in relationship with syphilis in 25 sera from the early stage of untreated natural human infection and in sera from 9 experimentally infected rabbits, collected periodically throughout 48 weeks of infection along with the effect of penicillin treatment in 5 rabbits of this group. At the same time, the sensitivities of these tests have been compared with the TPI and with non-treponemal tests (WR, KT, VDRL) with the same samples of serum, since the sensitivities of these latter tests in early human infection and in experimental rabbit syphilis are well documented. In this investigation, the relative sensitivities of the seven tests (WR, KT, VDRL, TPI, RPCF, RFTA and

FTA) at the different stages of syphilis are found to be as follows:

EXPERIMENTAL RABBIT SYPHILIS

(a) Early stage (0-8 weeks after infection) Table XII and Fig. 11)

(i) Non-treponemal tests (WR, KT, VDRL) - In spite of a weak non-specific reaction with KT in the control rabbit sera before infection, an increase of its titre (1:1.4) was noticed in the first week of infection along with the positive result of the VDRL test with the neat serum. In comparison, WR (Price's) became positive at a high titre (1:92) with the sera collected on the second week of infection. During the successive weeks, WR, VDRL and KT showed a descending order in sensitivity as was manifested by their titres. All these tests showed a double peak of antibody titres during this period and a decline in their titres was noticed in the later part of this period. Thus it could be concluded that amongst this group of non-treponemal tests, VDRL and KT showed positive results rather earlier in the infection than WR (Price's) although in the later period of this earlier stage, WR gave more strong positive reactions than KT and VDRL.

(ii) TPI test: The TPI test showed a weak titre (1:1.6) at a later period (fourth week) after infection in comparison

with the non-treponemal tests. The titre gradually increased in successive weeks. Thus the TPI test showed a poor reactivity at the beginning of infection in comparison with the non-treponemal tests although in the later part of this early stage, the titre was found to be high.

(iii) Newer treponemal tests:

RPCF - Although the RPCF test showed a non-specific reaction with the control rabbit sera at 1:9 dilution before infection, the titre of the test went up to 1:14 on the first week of infection showing a similar sensitivity to the KT and VDRL tests of the non-treponemal group and a more sensitive result than the TPI test. The titre of the RPCF test was much lower than the WR (Price's) in the second week of infection but on the sixth week, the titre showed more strongly positive results in comparison with all other tests used for this study except the FTA. After the sixth week of infection, the titre began to fall like the non-treponemal group of tests.

RFTA: Non-specific reactivities of the control rabbit sera before infection were also found with the RFTA test at a lower titre (1:2.6) in comparison with the RPCF and FTA tests. But the test showed a poor titre in the earlier period of infection in comparison with all the non-treponemal tests and with the FTA and RPCF tests of the newer treponemal group although the titre gradually increased in successive

weeks like the TPI and FTA tests.

FTA: Even with a non-specific reaction at 1:10 dilution with the control rabbit sera before infection, the titre of the FTA test went up to 1:120 on the first week of infection and showed the most sensitive result in comparison with the KT, VDRL, RPCF and RFTA tests which also showed reactivity on the first week. With the progress of infection, the FTA test manifested a gradual increase of its titre when the non-treponemal group of tests showed a decline in their titres along with the RPCF test. During this period of the early stage, the FTA test showed the highest sensitivity in comparison with all the tests used in this investigation.

Thus in the early stage of experimental rabbit syphilis, the relative position of the 7 serologic tests are found to be the FTA, RPCF, KT, VDRL, RFTA, WR and TPI in that order of sensitivity.

(b) Advanced stage (8-12 weeks following infection) Table XII, Fig. 11)

(i) Non-treponemal tests: All the tests (WR, KT, VDRL) in this group reacted at a high titre at this stage but WR showed the most strongly positive results, while KT was positive at a lower titre than the VDRL test. All these tests showed a decline in the titres in the later stage of the disease.

(ii) TPI test: The titre of the TPI test was lower than

the non-treponemal group in the earlier part of this stage and increased gradually throughout the whole period until it reacted at a higher titre than the non-treponemal tests from the 11th week of infection.

(iii) Newer treponemal tests: The RPCF test behaved similarly to the non-treponemal group in the gradual fall of its titre in the well developed stage of the disease although the reactivity was found at a much higher dilution of serum than with the non-treponemal tests.

RFTA: This test showed less sensitive reactions than the non-treponemal group in the early stage of the disease but in this advanced stage the test was found to be more sensitive than the latter group and behaved in sensitivity in the same way as the TPI test but at a much lower titre than the FTA test throughout this period.

FTA: This test showed the most sensitive results during this advanced stage of the disease reacting at 1:560 dilutions on the 8th week when WR showed a positive result at 1:56 dilution and on the 12th week of infection the test was positive at 1:1600 dilution when the TPI test reacted at 1:81 dilution. The gradual increase of the FTA titre during this period was similar to that of the TPI and RFTA tests.

To sum up, the results of this sensitivity study of 7 serologic tests in this well-developed stage of experimental

rabbit syphilis are found to be as follows:

(a) All the seven serologic tests showed a high titre at this stage of infection with the FTA at the top and the KT at the bottom of the list.

(b) Non-treponemal tests showed a gradual fall in their titre along with the RPCF test of the newer treponemal group but the TPI, FTA and RFTA tests showed a gradual increase of their titre during this stage of the disease.

(c) Late stage (12-48 weeks following infection) Treated Group of Rabbits (Table XII, Fig. 11)

(i) Non-treponemal tests (WR, KT, VDRL): Although the treatment affected the titres of these tests earlier along with the RPCF test of the newer treponemal group but the results of such effect varied with the different tests. Thus, the WR (Price's) became negative after 3 weeks, the titre of KT was lowered to its basic titre after 9 weeks and the VDRL became negative on the 18th week after completion of treatment. From this finding, it appear that in the post treatment period, the VDRL test showed the most sensitive result followed by KT and WR (Price's).

(ii) TPI test: The titre of the TPI test was least affected after the completion of treatment in comparison with

the other tests as a diminution of its titre was noticed only 6 weeks after completion of the treatment. The test was still positive after 36 weeks of treatment when all the other tests either showed a negative result or titres came down to their basic titres found in the control sera before infection.

(iii) Newer treponemal tests

RPCF: This test was affected in the same way as the non-treponemal group of tests (WR, KT, VDRL) after treatment. Its titre fell to the level of its basic titre (as was found in the control sera before infection) after 7 weeks of treatment and the test behaved similarly to the KT of the non-treponemal group in this treated late stage of infection.

RFTA: This test showed a fall of its titre just after completion of the treatment and was following by a sustained fall till the 16th week following treatment when the titre was similar to its non-specific titre as found in the control sera. Thus the RFTA test behaved like the non-treponemal group of tests after treatment but its sensitivity was much better than the WR, RPCF and KT (which became negative within 9 weeks after treatment) although it showed a less sensitive result than the VDRL test (as this test became negative on the 18th week following treatment).

FTA: The titre of the FTA test was affected by treatment much earlier than the TPI titre as a fall of titre

was manifested soon after starting of the treatment. But the FTA titre still remained high throughout the period of 24 weeks after treatment surpassing the titre of all other tests during this period and came down to its basic titre after a prolonged period of 26 weeks following treatment.

When the results of sensitivity of the 7 serologic tests are summarised in this treated group of rabb its, at the late stage of infection, it shows that WR, RPCF, KT, RFTA and VDRL tests became negative within 18 weeks of treatment in that chronological order. The FTA test showed positive results for the prolonged period of 26 weeks after treatment whereas the TPI test remained positive at a low titre (1:2. 2) till 36 weeks following treatment.

Untreated group of rabbits (Table XIII, Fig. 12)

(i) Non-treponemal tests: WR, KT and VDRL tests behaved differently in the untreated group of rabbits showing different degrees of sensitivity. The WR became negative on the 25th week of infection showing the least sensitive result whereas the VDRL manifested a weak positive result till the 48th week of infection showing the most sensitive result in this group. KT showed an intermediate sensitivity as this test manifested its reactivity similar to its non-specific titre (as was found incontrol sera before infection) on the 38th week of infection.

(ii) TPI test: The TPI test showed the most sensitive result amongst all the serologic tests used in this study as the titre was maintained at 1:243 dilution till the 48th week of observation.

(iii) Newer treponemal tests

RPCF: In this untreated group of rabbits, the RPCF titre was still positive at the 48th week of infection and showed more sensitive results than the VDRL test of the non-treponemal group at this 48th week of infection, although the test showed a lower sensitivity in comparison with the TPI and FTA tests as the titre of the RPCF was much lower than these two tests at the 48th week.

RFTA: This test of the newer treponemal group showed similar sensitivity to KT as its titre fell to its basic level of non-specific titre (as was found in the control sera) on the 38th week of infection. Thus the test showed a poor sensitivity in comparison with the TPI, FTA, RPCF and VDRL tests which remained positive till the 48th week of infection but was found to be more sensitive than the WR which became negative earlier (on the 25th week of infection).

FTA: Although the titre of the FTA test diminished to a great extent throughout the late stage of infection, it still showed apositive result of 1:200 dilution of sera at the end of the 48th week of infection. This titre is much higher

than its non-specific titre (1:10) which was found with the control sera before infection. This titre of 1:200 of the FTA test at the end of 48 weeks in this untreated group of rabbits compares favourably with the TPI titre (1:243) and is much higher than the titre of RPCF and VDRL tests which also showed positive results till the 48th week of infection.

When the sensitivity of the 7 serologic tests are summarised in this untreated group of rabbits at the late stage of infection, their relative positions are TPI, FTA, RPCF, VDRL, RFTA, KT, WR in that order of superiority.

Besides the FTA and RFTA tests, the pattern of antibody titres with other serologic tests in experimental rabbit syphilis was also studied in the past by various workers like Chacko (1953), Turner & Hollander (1957) and Pavlatou et al. (1960). These workers were of the opinion that at the beginning of infection, the non-treponemal tests as well as the RPCF test were more sensitive than the TPI test whereas in the late untreated stage of the disease, the TPI and RPCF tests remained positive when the non-treponemal tests showed negative results. Treatment had little effect on the TPI titre unless instituted in the earlier part of the infection although the titre of the non-treponemal tests declined rapidly after treatment.

The results of this present study regarding the antibody patterns with the RPCF, TPI and non-treponemal tests in experimental rabbit syphilis are found to be similar to the reports of these workers but so far the sensitivity of the FTA and RFTA tests in experimental rabbit syphilis has not been reported.

This difference in the reactivities of the different serologic tests in the treated and untreated groups of rabbits is not due to the production of a systemic lesion in the latter group of rabbits as no such lesions were found after histological examination of the different organs from the post-mortem material on the 48th week of infection. Even the testes showed normal appearance after 48 weeks (Fig. 14a, b; Fig. 15a, b) in both groups of rabbits but when the section of testis from another rabbit was examined after one month of infection, it showed an interstitial granulomatous reaction (Fig. 13a, b). This localised nature of the disease in experimentally infected rabbits was also reported earlier by other workers (Browne & Pearce, 1920; Hollander & Turner, 1954).

Thus the difference of serologic reactivities in the two groups of treated and untreated rabbits could be explained as due to the difference in time of persistence of the treponemes in the rabbit testes. Treponemes are killed earlier in the treated group by penicillin whereas in the untreated group

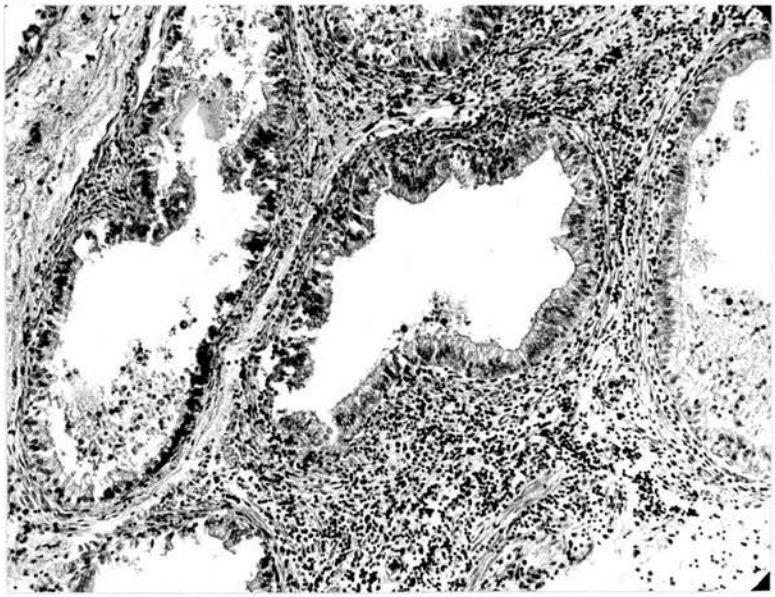

Fig. 13a. Syphiloma of rabbit testis one month after intratesticular injection of T. pallidum. H and E stain x 120.

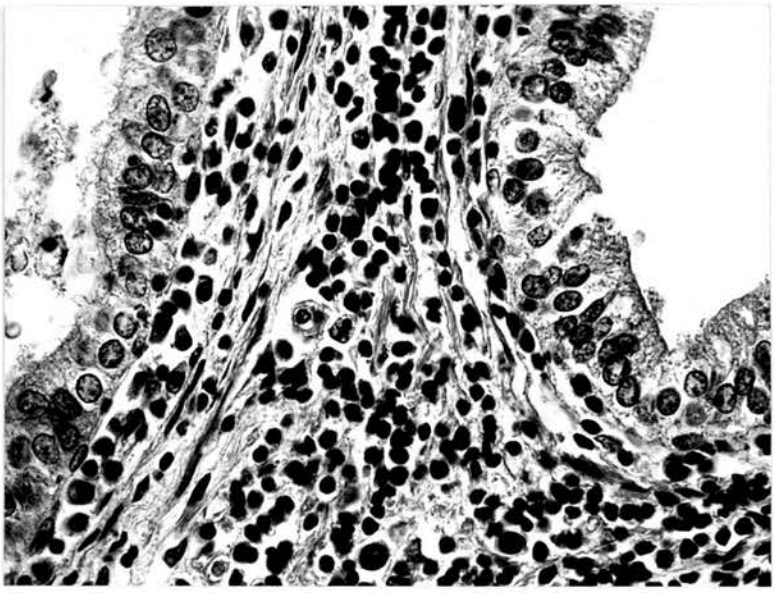

Fig. 13b. Syphiloma of rabbit testis, one month after intratesticular injection of T. pallidum. H and E stain x 500.

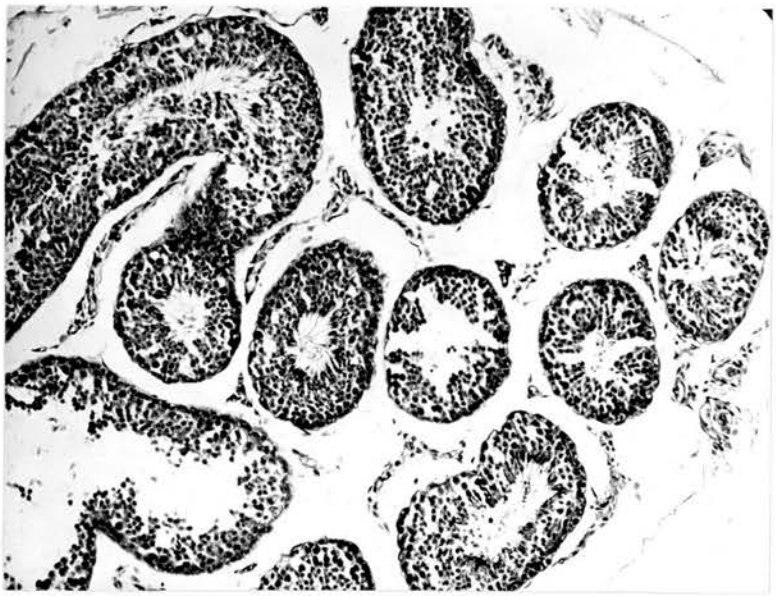

Fig. 14a. Section of rabbit testis showing disappearance of inflammatory reaction 36 weeks after treatment. H and E stain x 120.

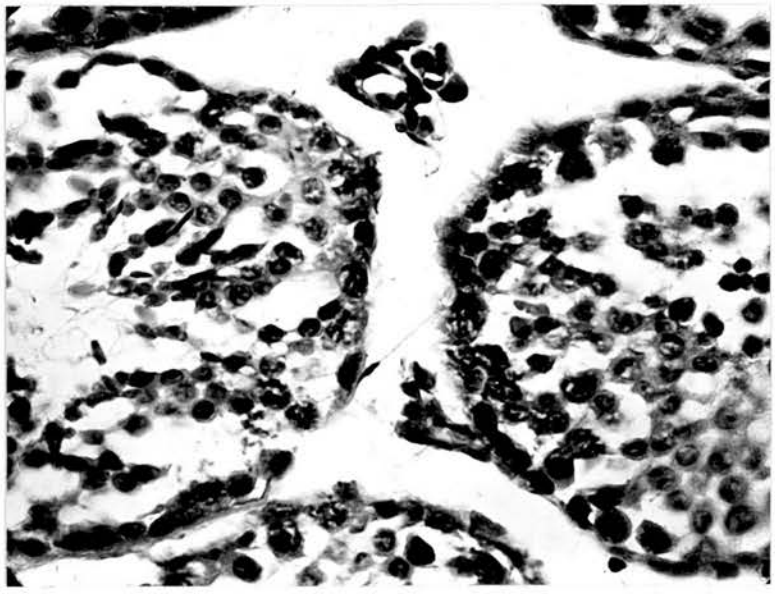

Fig. 14b. Section of rabbit testis showing disappearance of inflammatory reaction 36 weeks after treatment. H and E stain x 500.

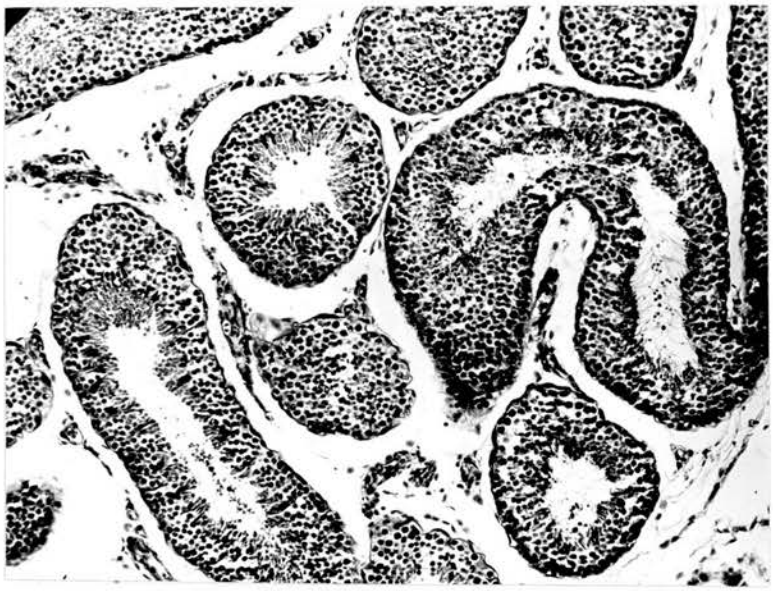

Fig. 15a. Section of rabbit testis showing disappearance of inflammatory reaction one year after intratesticular injection of T. pallidum. H and E stain x 120.

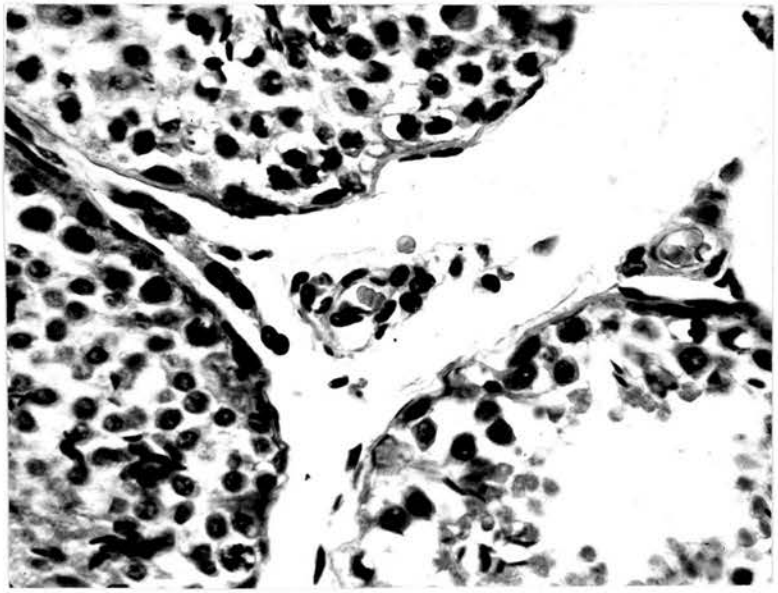

Fig. 15b. Section of rabbit testis showing disappearance of inflammatory reaction one year after intratesticular injection of T. pallidum. H and E stain x 500.

the treponemes are killed after a long time by the systemic definsive mechanism of the rabbit. The antibody produced in these two group of rabbits varied due to prolonged stimulation of the R. E. system by the treponemes in the untreated group but the stimulus was only for a short period in the treated group of rabbits.

EARLY UNTREATED PRIMARY HUMAN SYPHILIS

TABLE XXa Comparative sensitivity of 7 tests in 25 sera from early untreated human syphilis

Tests	No. tested	Positive	Sensitivity (%)
WR (Price's)	25	16	64
KT	25	16	64
VDRL	25	16	64
TPI	25	11	44
RPCF	25	16	64
FTA-200	25	18	72
RFTA-200	25	0	0

From the results (Table XIV) of the different serologic tests (WR, KT, VDRL, TPI, RPCF, FTA-200, RFTA-200) with the sera from early untreated human syphilis, Table XXa has been obtained. This table shows the comparative sensitivity of the different serologic tests in this group of sera. The FTA-200 test showed the highest sensitivity in comparison with the WR, KT, VDRL and RPCF tests, which behaved similarly and stood together next to FTA-200 in their

sensitivity. The TPI test showed a poor sensitivity in this group, reacting with 44% of the sera in comparison with 72% reactivity of the FTA-200 test. The RFTA test did not react with any of the sera at 1:200 dilution showing the least sensitive result.

This result of higher sensitivity of the RPCF test over the TPI in the early stage of untreated human syphilis also confirms the result of Foster et al. (1959). The findings of earlier reactivity of the FTA-200 test in comparison with the other tests in the sera from early human cases of untreated syphilis also supports the earlier findings of Wilkinson (1961). But the findings of the RFTA-200 reactivity with this group of sera do not correspond with the observations of Scott et al. (1961) who considered the test to be similar in sensitivity to the FTA-200 test in the early stage of syphilis. The lag in appearance of the TPI reactivity in early primary cases of syphilis prevents its use as a diagnostic test in these cases (Magnuson & Thompson, 1949; Mohr et al., 1950; Nelson et al., 1951; Durel et al., 1951; Chacko, 1953; Edmudson et al. 1954; Wilkinson, 1954; Sequeira, 1955; Neilsen & Rein, 1956).

Attempts were made to increase the sensitivity of the FTA-200 test by lowering the testing dilition of the sera at 1:25 so that it will react with all the sera from the early stage of syphilis as only 18 cases showed positive results

out of 25 sera used for this study (Table XIV). Table XV shows that when the FTA test is used at 1:25 dilution of serum all the non-reactive FTA-200 sera from early cases of syphilis became positive at this dilution whereas at this 1:25 dilution all the 25 normal sera showed negative results.

The relative sensitivity of each test in the different stages of syphilis could be expressed as follows:

(i) Non-treponemal tests:

WR (Price's): In the early stage of the disease, this test showed a more sensitive result than the TPI in the experimental rabbit syphilis whereas in the early cases of human infection, the test was similar to the KT, VDRL, RPCF tests in its sensitivity and showed a more sensitive result than the TPI and RFTA-200 tests.

In the advanced stage of experimental rabbit syphilis, this test was sensitive enough to compete with other tests and showed a better result than the KT.

In the late stage of experimental rabbit syphilis, WR (Price's) showed poor sensitivity in both the treated and untreated group of rabbits in comparison with other tests.

KT: Although the test showed non-specific reaction with the control sera before infection, it produced a more sensitive result in the early stage of the experimental rabbit syphilis in comparison with the WR and TPI tests whereas in

early cases of human syphilis, this test was similar in sensitivity to WR, VDRL and RPCF tests and was more sensitive than the TPI and RFTA-200 tests.

In the advanced stage of experimental rabbit syphilis, the test was the least sensitive amongst the 7 serologic tests included in this study.

In the late stage of experimental rabbit syphilis, KT showed more sensitivity than the WR and RPCF tests in the treated group of rabbits whereas this test behaved similarly to the RFTA tests and showed more sensitive reactions than the WR in the untreated group of rabbits.

VDRL: In the early stage of experimental rabbit syphilis, this test behaved better than the WR and TPI tests in its sensitivity but in early cases of human infection, VDRL showed similar sensitivity to the WR, KT and RPCF tests in detecting these cases and reacted better than the TPI and RFTA-200 tests.

In the advanced stage of the experimental disease in rabbits, the VDRL test behaved similarly to most of the serologic tests in showing a high titre and showed better results than the KT.

In the late stage of experimental rabbit syphilis, the VDRL test was more sensitive than WR, RPCF, KT and RFTA tests in the treated group whereas this test remained positive

in the untreated group till the 48th week of infection when the TPI, FTA and RPCF tests also showed positive results.

TPI: The TPI test showed the least sensitive result in the early stage of both human and experimental rabbit syphilis although the test showed much better results than the RFTA-200 tests in the former group.

In the advanced stage of rabbit syphilis, the TPI test showed a reasonable sensitivity along with the other tests but reacted better than KT (as KT showed reactivity at a much lower titre) and showed a less sensitive result than the FTA test which was positive at a much higher titre.

In the late stage of the disease in rabbits, the TPI test showed the best result in the untreated group remaining positive at a high titre (till 48 weeks of infection) in comparison with the FTA, RPCF and VDRL tests. In the treated rabbits, the test showed little effect of treatment indicating a positive result on the 38th week after treatment when all other tests showed negative results including the FTA test.

RPCF: In spite of a non-specific reaction with the control sera in rabbits, this test showed more sensitive results in the early stage of experimental syphilis than the WR and TPI tests whereas in early human cases of syphilis, this test compared well with the WR, KT and VDRL tests and showed a more sensitive result than the TPI and RFTA-

-200 tests.

In the advanced stage of syphilis in the rabbit, the RPCF test could be regarded as similar in sensitivity to the WR, VDRL, TPI and RFTA tests.

In the late stage of infection in rabbits, the RPCF reactivity disappeared earlier in the treated group of rabbits in comparison with the KT, VDRL, RFTA, FTA and TPI tests whereas in the untreated group, this test behaved like the VDRL, TPI and FTA tests in showing positive results till the 48th week of infection, although the titre was much lower than the FTA and TPI tests at this time.

RFTA: This test showed a sensitivity similar to the RPCF, KT, VDRL in the early stage of experimental rabbit syphilis but in the case of early human syphilis, the RFTA-200 test showed the poorest result in comparison with the other tests when performed at 1:200 dilution of serum as was recommended by the authors of the test.

In the advanced stage of the disease in the rabbit, the RFTA test showed similar sensitivity to the WR, VDRL, RPCF and TPI tests and showed a more sensitive result than KT.

In the late stage of the disease in experimental rabbit syphilis, the RFTA test showed better results than the WR, KT, RPCF tests as this test maintained its reactivity till 15

weeks after treatment.

In the untreated group, the test reacted similarly to KT and showed a better result than the WR in its sensitivity as the RFTA test was reactive till the 37th week of infection.

FTA: This test showed the most sensitive result in the early stage of rabbit syphilis but in the early human infection, the FTA-200 test also showed similar sensitive results and became positive earlier than the sensitive non-treponemal group of tests.

In the advanced stage of the disease in rabbits, the FTA test also surpassed the titre of other tests and showed its maximum sensitivity.

In the late stage of experimental rabbit syphilis, the FTA test was reactive for a prolonged period of 26 weeks in the treated group of rabbits in comparison with the other tests (excluding the TPI), which showed less sensitive results as they become negative earlier whereas the TPI test showed its reactivity till 36 weeks after treatment. In the untreated group, the FTA test showed a poor sensitivity similar to the TPI test and was found to be more sensitive than the RPCF and VDRL tests on the 48th week of infection.

Thus it is clear from this observation that so far as sensitivity is concerned in all stages of experimental rabbit syphilis (treated and untreated groups) 7 serologic tests

behaved in the following order: 1. FTA 2. RPCF 3. VDRL 4. TPI 5. KT 6. RFTA 7. WR whereas in early untreated human syphilis the tests behaved rather differently, the order of sensitivity being - 1. FTA-200 2. WR, KT, VDRL, RPCF 3. TPI 4. RFTA-200.

Evaluation of specificity

1. Biologically false positive sera: In this investigation, the specificity of the 7 serologic tests (WR, VDRL, KT, TPI, RPCF, FTA-200, RFTA-200) were studied in a group of 57 biologically false positive sera collected from 3 different sources as shown in Tables XVI, XVII and XVIII. These 57 sera included 23 samples from normal healthy blood donors after repeated donation, 25 samples from cases of rheumatoid arhtritis and the rest consisted of a miscellaneous group including nephrotic syndrome, pulmonary tuberculosis, glandular fever, systemic lupus erythematosus, pregnancy and erythema nodosum. The inclusion of these cases for the study of the specificity of serologic tests is mainly due to the regular appearance of antilipoid antibody (reagin) in these sera which reacts with the lipoid antigens used for the test. The mode of production of reagin varies with the disease concerned. In infectious diseases like tuberculosis where destruction of tissue is a prominent feature, the reagin appears in the serum as a result of stimulus by the tissue

lipoid component, (Sequeira, 1959) whereas in connective tissue diseases like systemic lupus erythematosus and rheumatoid arthritis, the reagin is one of the manifestations of the wide variety of antibodies produced in such cases (Raffel, 1961). Thus it is clear that when non-treponemal tests are used, there is more chance of obtaining non-specific reactions with these sera than when treponemal antigens are used for the test although the latter antigen also has a minor lipoidal component. The specificity of other serologic tests are judged from the reactivity of the TPI test considering it as a specific standard test for syphilis because the antilipoidal antibody has no effect on the immobilisation of treponemes.

It is clear from Table XIX that the specificity of the newer treponemal group of tests is better than that of non-treponemal tests using lipoidal antigen. Both the FTA-200 and RFTA-200 tests of the newer treponemal group showed a specificity of 100%. This finding of absolute specificity with the FTA-200 test is in agreement with the findings of earlier workers like Wilkinson (1961), Mannucci and Spagnoli (1961). But when the specificity of the FTA-25 test was studied with the sera from this group of biologically false positive reactors (Table XX) this test showed positive results with most of these sera. Thus it is apparent from this result that the specificity of the FTA-200 test is lost to a marked extent

after lowering the testing dilution of the serum from 1:200 to 1:25.

The 100% specificity of the RFTA-200 test may be related to its poor sensitivity as this test also showed negative results at 1:200 dilution with all the 25 sera from early cases of human syphilis (Table XV) although the specificity of the RFTA-200 test was claimed as similar to the FTA-200 test by the authors (Scott et al. 1961).

As the antigen used for the RPCF test is claimed as a purified protein antigen, one should expect a specificity of 100% like the TPI test but the result in this study shows a specificity of 91.3% contrary to expectation. A similar finding of non-specific reactions with the RPCF test was also reported by Berner et al. (1960).

Although the non-treponemal group of tests showed a fair number of non-specific results with the 57 biologically false positive sera, the reactivity varied with the type of antigen used and the nature of the test performed. Thus when the complement fixation test (WR Price's) was performed, it showed a specificity of 79% whereas in the flocculation test (KT) with the similar crude heart extract antigen and the same samples of sera, the result showed a specificity of 0.0%. The use of synthetic cardiolipin antigen in the flocculation test (VDRL) changed the specificity to 45.5%.

Thus these results indicate that the 7 serologic tests behaved in their specificity in the following order: 1. TPI, FTA-200 2. RPCF 3. WR (Price's) 4. VDRL 5. KT. The result of the RFTA-200 test is inconclusive.

2. Normal rabbit sera (Table XII, XIII): Sera from most of the rabbits showed a non-specific reaction with KT, RPCF, FTA and RFTA tests before infection. The non-specific reactivity with KT, FTA, AND RFTA tests could be explained as being due to a reagin which may be present in rabbit serum (presence of reagin in the sera of other animals has also been reported by Kemp et al., 1940) and it reacts with the lipoidal antigenic fractions present in these curde hearth extract and treponemal antigens. The non-specific reactivity in these sera with the RPCF test cannot be explained as the antigen is a purified protein and the rabbits were free from any other infections like coccidiomycosis or rabbit syphilis (T. cuniculi) as no evidence of such diseases was found in histological examinations of different organs after autopsy.

When both the sensitivity and specificity of these 7 serologic tests are considered, they are found to be in the following order:

(1) FTA-200 test: This test is found to be the most sensitive amongst all the 7 serologic tests used in this study for the diagnosis of early cases of human infection.

In experimental rabbit syphilis, the FTA test is more sensitive than all the other tests in early and advanced stages of the disease. In the late untreated stage, the reaction is as sensitive as the TPI test whereas in the late stage of treated rabbit syphilis, the FTA test becomes negative some time after cessation of treatment and could be used as a test for cure.

Like the TPI test, FTA-200 could eliminate biologically false positive reactions.

(2) TPI test: Although this test is less sensitive than other tests (used in this study) in early primary cases of human syphilis and in the earlystage of syphilis in the rabbit, it shows a sensitive result like all the other tests in advanced stages of the disease in rabbits. In late untreated rabbit syphilis, the test remains positive at a high titre but in the case of treated late syphilis, the TPI test has little value in judging the effect of treatment as it remains positive indifinitely.

The TPI test eliminates biologically false positive reactions as it is universally accepted as a specific test for syphilis.

(3) RPCF test: Shows as sensitive results as the non-treponemal group of tests in early cases of human syphilis and manifests a better result than WR (Price's) in the early stage of experimental disease in rabbits.

The RPCF test is as sensitive as other tests in the advanced stage of rabbit syphilis and the test remains positive in late untreated rabbit syphilis for a prolonged period like the TPI and FTA tests. In treated rabbits, it becomes negative earlier in comparison with the FTA test.

The RPCF test also shows a better degree of specificity than the non-treponemal group of tests.

(4) VDRL test: shows a sensitive result like the other treponemal tests in early cases of human syphilis but in the early stage of experimental rabbit syphilis, it is found to be more sensitive than the WR (Price's). VDRL manifests sensitive results similar to other tests in the advanced stage of the disease in rabbits and remains positive for a long time like the RPCF test in untreated cases, whereas in the treated late case of rabbit syphilis, the test becomes negative earlier than the FTA test.

The non-specific result revealed by this test is less than the KT but surpasses the results of WR (Price's).

(5) WR (Price's): This test shows sensitive results like the other non-treponemal tests in early cases of human syphilis but in the early stage of experimental rabbit infection, WR is found to be less sensitive than the KT and VDRL tests. It behaves like all other tests in sensitivity in the advanced stage of syphilis in rabbits but in the late stage of the disease,

WR becomes negative at first in comparison with other tests in both the treated and untreated cases.

WR shows a less non-specific result than the KT and VDRL tests.

(6) KT: Although this test shows sensitive results in early cases of human syphilis like the other non-treponemal tests and manifests more sensitive results than the WR (Price's) in the early stage of experimentally infected rabbits, it is found to be less sensitive than other tests in the advanced and late stage of the disease in rabbits (treated and untreated).

KT shows the maximum non-specific reactions amongst all the serologic tests used for this study.

(7) RFTA-200 test: This test at 1:200 dilution is the least sensitive amongst all the serologic tests used for this study as the test showed negative results with all the 25 sera of early human syphilis used for this investigation. So the question of non-specific reaction does not arise at all. If the test is used for diagnostic purposes at a much lower dilution of serum, there is every likelihood of getting results like the non-treponemal group of tests, as the RFTA test was found to be closer in sensitivi ty to the latter group of tests in all the stages of experimentally infected rabbits (except the VDRL test in the untreated group).

PART II

Study of serologic reactivity of serum protein fraction.

Chapter VI

METHODS

Chromatographic fractionation of serum protein by DEAE-Sephadex column

Principle

DEAE-Sephadex (Pharmacia, Uppsala, Sweden), an anion exchange resin, is obtained by the introduction of an ionic group (diethyl-aminoethyl) into Sephadex, which in itself consists of small grains of a hydrophilic insoluble substance made by cross-linking the polysaccharide dextran. By variation in the degree of cross linkage of polysaccharide different porosities of the network are obtained. Thus DEAE-Sephadex A-50 variety is less cross linked but has a high porosity and the ionic groups are accessible to much larger molecules like serum protein globulin. The protein molecules are absorbed by the ionic groups at a pH of 6.6 suspending buffer solution and complete elution of the absorbed protein is generally made by changing the ionic strength of the surrounding medium.

Reagents

(a) Sodium phosphate buffer (0.2 M) pH 6.6

Composition:

Solution A: NaH_2PO_4, $2H_2O$ - 31.2 gms/1000 ml.

Solution B: Na_2HPO_4, $12H_2O$ - 71.79 gms/1000 ml.

62.5 ml. of solution A + 37.5 ml. of solution B

pH is adjusted to 6.6

(b) Sodium chloride solution

(i) 0.14 M. - 8.199 gms. NaCl/1000 ml.

(ii) 0.34 M. - 19.89 gms. NaCl/1000 ml.

(iii) 0.76 M. - 44.46 gms. NaCl/1000 ml.

(c) .5 N Hcl solution

(d) .5 N NaOH solution

(e) NaH_2PO_4 (.2M) solution: NaH_2PO_4, $2H_2O$ - 31.2 gms/1000 ml.

(f) distilled water

Preparation of DEAE-Sephadex before using in column

The exchanger was prepared for use as follows:

1. 1 gm. of DEAE-Sephadex A-50 (medium) (sufficient for fractionation of 2 ml. serum) measured in a conical flask and allowed to swell up with excess distilled water in a refrigerator (4^o - 6^oC).

2. Excess water was decanted by suction to remove the fine particles.

3. The exchanger was then put on two sheets of Whatman (No. 40) filter paper in a Buchner funnel for washing with different solutions. A suction pump was used for washing at

a negative pressure of 15 lbs. Washing was carried out stepwise in the following manner:

(a) 250 ml. of .5 N Hcl.

(b) 250 ml. of distilled water

(c) 250 ml. of .5 N NaOH

(d) 250 ml. of distilled water

(e) 250 ml. of NaH_2PO_4 (0.2M) solution

(f) 250 ml. of NaH_2PO_4 (0.02M) solution

(g) .02 M sodium phosphate buffer (pH 6.6)

Washing with buffer solution was continued until the pH of washed out buffer solution became 6.6.

Preparation of column for fractionation

The apparatus used was as shown in the diagram (Fig. 16). Washed DEAE-Sephadex suspended in .02 M. Sodium phosphate buffer solution was poured into the glass tube (with tapering end) with a pad of glass wool at the bottom and was allowed to settle in a refrigerator (4^0 - 6^oC) for 3 days before using it for fractionation.

Procedure for fractionation of serum protein

1. 2.5 ml. of serum was dialysed against 100 ml. of .02M solium phosphate buffer (pH 6.6) overnight in a refrigerator (4^o - 6^0C).

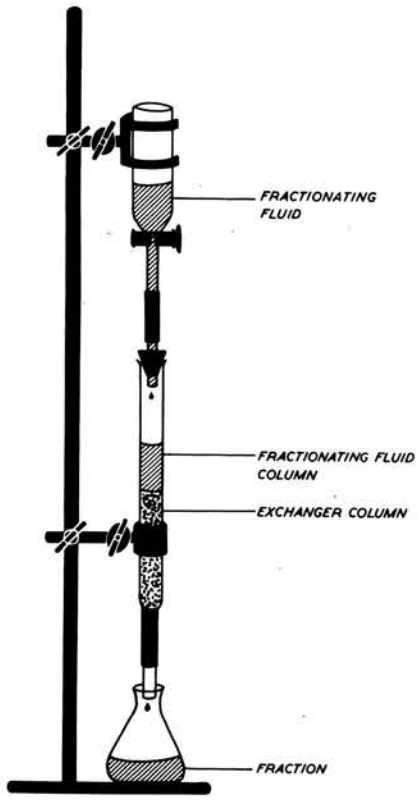

Fig. 16.

The apparatus used for chromatographic fractionation of sera.

2. 2 ml. of dialysed serum was pipetted on the buffer column inside the fractionation tube.

3. Different solutions were allowed to run through the column slowly (12 drops per minute approx.) and were collected from the bottom of the column in conical flasks in the following order.

FRACTION 1: .02M Sodium phosphate buffer pH 6.6 - 100 ml.

FRACTION 2: same as fraction 1

FRACTION 3: 0.2M Sodium phosphate buffer solution (pH 6.6) - 10 ml.
Distilled water- 40 ml.
0.14 M.NaCl - 50 ml.
TOTAL 100 ml.

FRACTION 4: Same as fraction 3

FRACTION 5: 0.2M Sodium phosphate buffer solution (pH 6.6) - 10 ml.
Distilled water 40 ml.
0.34 M. NaCl - 50 ml.
TOTAL 100 ml.

FRACTION 6: Same as fraction 5

FRACTION 7: 0.2M Sodium phosphate buffer solution (pH 6.6) - 10 ml.
Distilled water 40 ml.
0.76 M NaCl - 50 ml.
TOTAL 100 ml.

Estimation of protein content of different fractions

5 ml. of each fraction was collected from each flask for estimation of protein content by measuring optical density in an UnicamSpectrophotometer with hydrogen lamp at wavelengths of 280 mμ and 260 mμ. The final concentration of protein was calculated from a nomogram. The total amount of serum protein was then calculated from the fractions and compared with that present in original serum.

TABLE XXI:
RESULTS OF PROTEIN ESTIMATION OF THE DIFFERENT FRACTIONS OF A RABBIT SERUM IN RELATION TO ITS PROTEIN CONTENT

Serum sample no. B_1 (4)

Fractions	Reading at 280 mμ	Reading at 260 mμ	Percentage of protein mg/ml.	Amount in ml.	TotalProtein content in mg.
1	.105	.072	.11	100	11
2	.03	.022	.03	100	3
3	.242	.158	.25	100	25
4	.016	.005	.013	100	1.3
5	.36	.21	.4	100	40
6	.056	.05	.04	100	4
7	.066	.042	.07	100	7
Serum (diluted 1:1000)	.095	.056	.1	2ml.	Tot. 91.3mg. / 200 mg.

Concentration of fractions

The fractions were then poured into dialysis tubings. The tubings were surrounded by carboxymethylcellulose powder, under a constant weight, in a cool room (4° - 6°C) for a period of 3 days. Carboxymethylcellulose was changed once in 24 hours and the tubings were tightened at the same time to hasten the concentration procedure. Thus the fractions were concentrated to the original volume (2 ml.) (It was necessary to concentrate both the biologically false positive and the weak reactive syphilitic sera to 1 ml. as the titre was low).

Dialysis of concentrated fractions

As the concentrated fractions contained high molar concentration of NaCl these were dialysed against .02 M sodium phosphate buffer solution overnight in a refrigerator (4°C - 6°C) to drive out the excess salt so that specimens might be used in serologic tests.

Electrophoretic study of different fractions

Electrophoresis was performed in Shandon electrophoretic instrument on cellulose acetate paper (size 20 cm. x 5 cm. No. 50. Oxoid, Oxo Ltd., London E. C. 4) for 2 hrs. in veronal buffer solution (pH 8.6) at 12 V/cm.

Veronal buffer solution

Solution I (a) Sodium barbiturate - 10.3 gms.

(b) Barbituric acid - 1.84 gms.

(c) Distilled water - 1000 ml.

Solution II (a) Boric acid solution - 350 ml.

Boric acid - 15.46 gm.

Distilled water - 500 ml.

(b) NaOH solution - 100 ml.

NaOH - 5 gm.
Distilled water - 250 ml.

(c) Distilled water - 675 ml.

Equal volumes of solution I and II were mixed and pH was adjusted to 8.6.

The cellulose acetate paper strips were dried in a hot air oven (100°C) for 20 minutes and were stained in Nigrosine solution overnight.

Nigrosine solutions: Nigrosine .0025 gm.

Glacial acetic acid 2 ml.

Distilled water 98 ml.

The strips were washed in tap water and were allowed to dry after blotting off escess water.

As most of the electrophoretic and serologic activity of serum were confined in fractions 1, 3, 5 and 7 and no serologic activity was found even after testing combined fractions (e.g.

1 + 2, 2 + 3 and so on) in biologically false positive sera, other fractions were discarded throughout the present study after estimation of protein content.

Characterisation of serum protein fractions

Serum protein fractions were identified by their electrophoretic mobility in comparison with original serum and were recorded as follows:

(a) Human serum (Fig.17)

Fraction 1 - $Gamma_2$ globulin (or 7s type of globulin)

Fraction 3 - $Gamma_1$ or $Beta_2$ M globulin (including Macroglobulin or 19s type of globulin)

Fraction 5 - Mixture of $alpha_1$, $alpha_2$ globulin, albumin and a trace of gamma gl obulin.

Fraction 7 - Mixture of $beta_2$ and alpha2 globulin and a trace of gamma globulin.

(b) Rabbit serum (Fig.18)

Fraction 1 - $Gamma_2$ globulin (or 7s type of globulin)

Fraction 3 - $Gamma_2$ globulin (or 7s type of globulin) and $gamma_1$ globulin or $beta_2$ M globulin (including Macroglobulin or 19s type of globulin)

Fraction 5 - Mixture of $alpha_1$, $alpha_2$ globulin, albumin and a trace of gamma globulin.

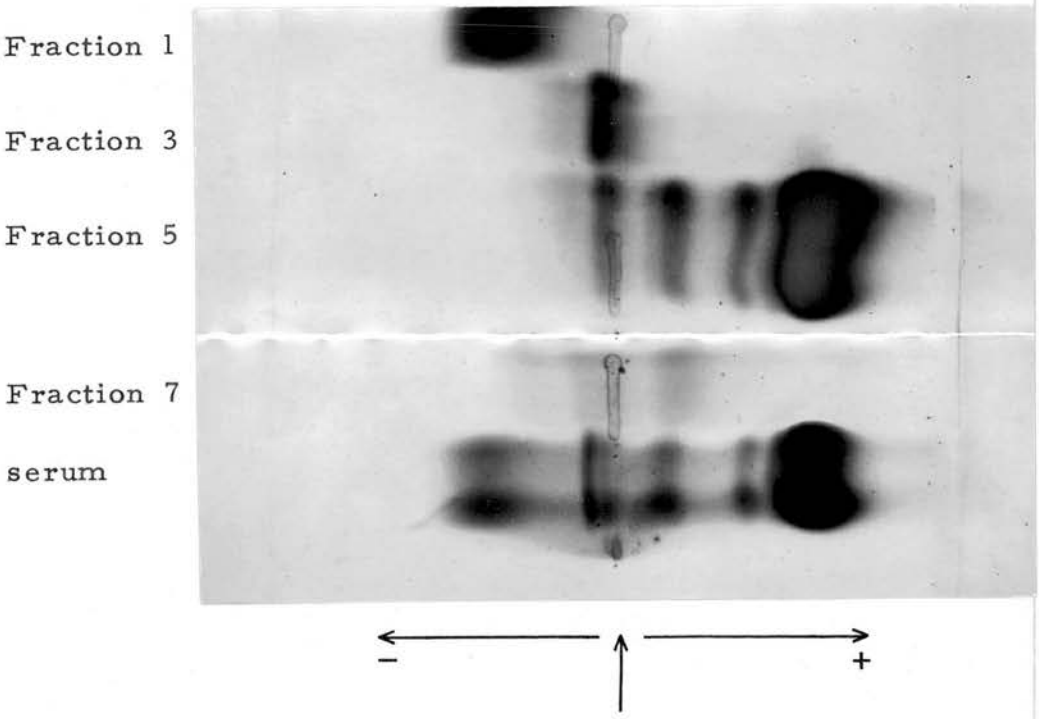

Fig. 17. Electrophoresis of a human serum and its different fractions on cellulose acetate paper.

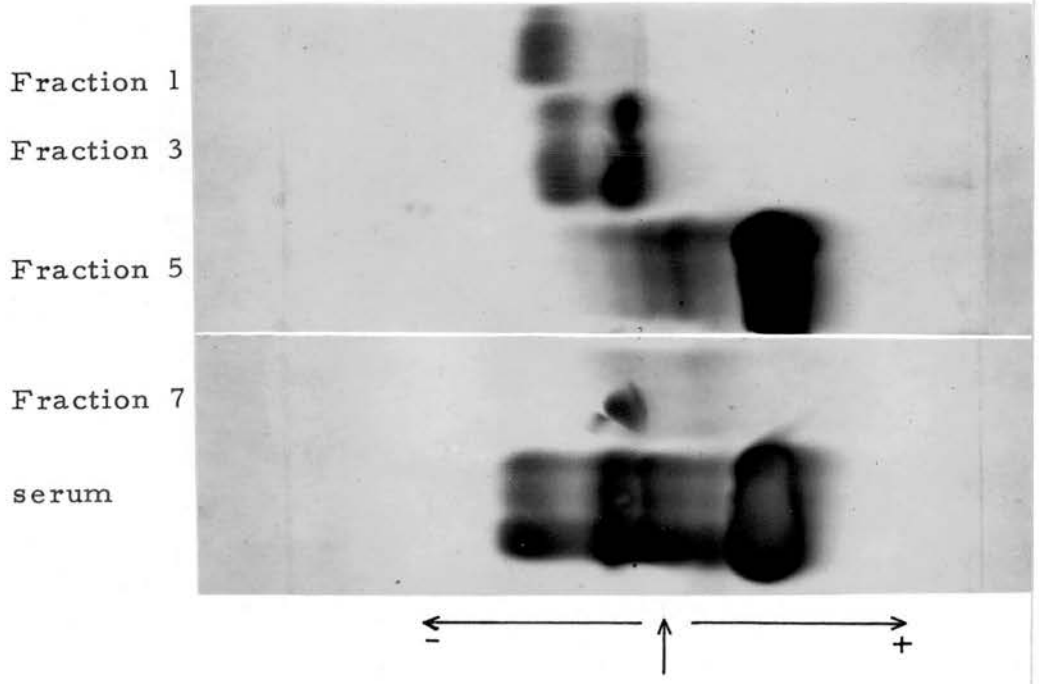

Fig. 18. Electrophoresis of a rabbit serum and its different fractions on cellulose acetate paper.

Fraction 7 - Mixture of $beta_2$, $alpha_2$ globulin and a trace of gamma globulin.

Tests for purity of each serum protein fraction:

Purity of fractions 1 and 3 from the human serum was analysed by immunoelectrophoresis in agar gel against Coombs serum (Fib.19 b, c). In this reaction fraction 1 was found to be one single purified component (Fig.19b), whereas fraction 3 showed a combination of two antigenic constituents (Fig.19c), one prepondering over the other. The second component, showing a weak precipitation line, corresponds to $gamma_2$ globulin. Such combination was also found in fraction 3 of the rabbit serum (Fig.18) although both the components in this fraction showed similar concentration.

Attempts were made to separate the two components in fraction 3 by destroying the $gamma_1$ globulin (macroglobulin) with mercaptoethanol (Grubb and Swahn, 1958), by splitting the linkage bond between the two components by urea hydrolysis followed by starch block electrophoresis and differential elution, by repetition of the same process of fractionation before and after urea hydrolysis. But none of these methods helped in the separation of the two components as the agar gel diffusion precipitation tests (Fig.20) of these treated materials with the Coombs serum still showed two such com-

a.

b.

c.

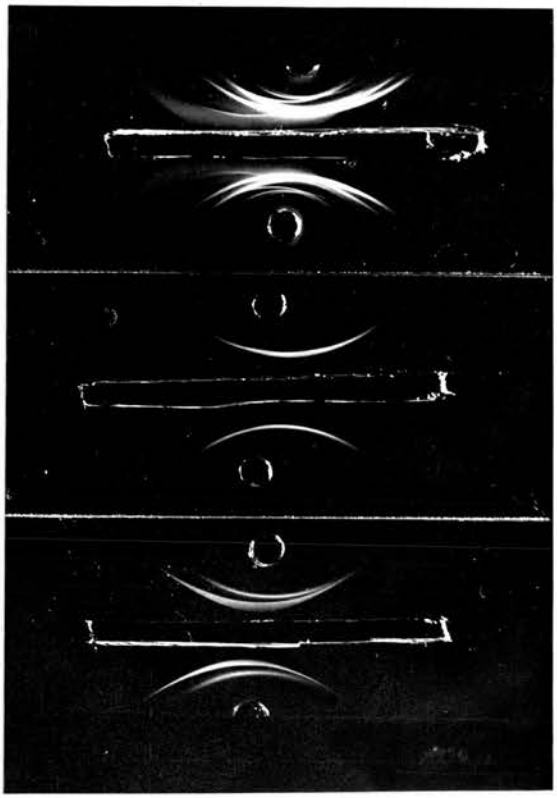

Fig. 19 Agar gel immunoelectrophoresis of human serum (a), fraction 1, (b) and fraction 3, (c) of the same serum sample (in the wells) against antihuman globulin (Coombs) serum in all the gutters for a check in purity of the serum fractions.

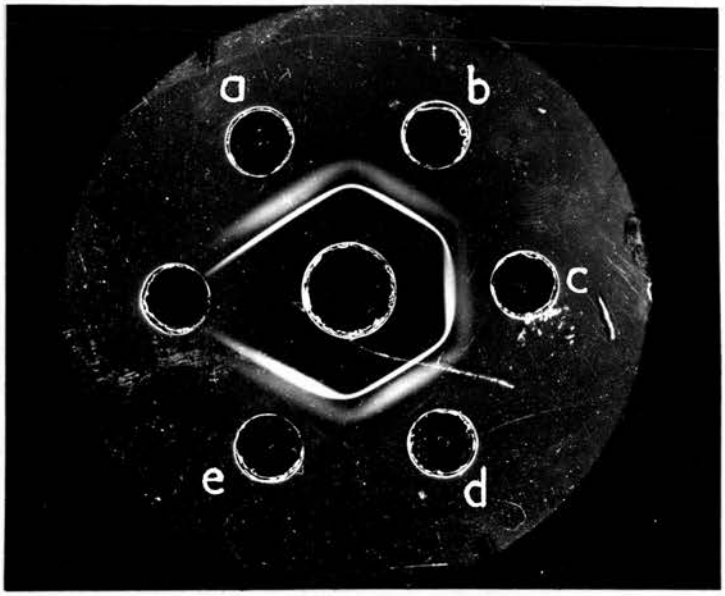

Fig. 20 Agar gel diffusion precipitation reactions of fraction 3 from human serum before and after treatment with different chemicals and refractionation (peripheral wells) against antihuman globulin (Coombs) serum (central well).

Wells:
- a. Fraction 3 without any treatment.
- b. Fraction 3 treated with mercapto-ethanol.
- c. Fraction 3 hydrolised by urea and eluted after starch block electro-phoresis.
- d. Fraction 3 after repetition of fractionation through exchanger column.
- e. Fraction 3 hydrolised by urea and repetition of fractionation through exchanger column.

ponents although the reactivity of the second component in the urea treated fractions showed fainter precipitation lines in comparison with the others (Fig. 20 c, e).

Besides their individual component of globulin, fractions 5 and 7 were found to be mixed with a trace of gamma globulin as judged from their electrophoretic character. Such contamination with gamma globulin raised some doubt about the serologic reactivity of the individual component in fraction 5 and 7 when these fractions showed positive reactions in different stages of syphilis. It was presumed that the contaminant gamma globulin was responsible for the seroreactivities of such fractions. So attempts were made to characterise the particular globulin responsible for serologic reactivity in fractions 5 and 7. Serum fractions (5 and 7) from the advanced stage of the disease (when both the fractions showed seroreactivity) were tested for their gamma globulin content by quantitative agar gel diffusion technique (Fig.21a, b). (Feinberg, 1957) against antihuman fraction II gamma globulin (Nutritional Biochemical Corporation, Cleveland, Ohio), prepared in rabbit. The antiserum showed activity mainly against human gamma globulin in immune electrophoresis (Fig.22).

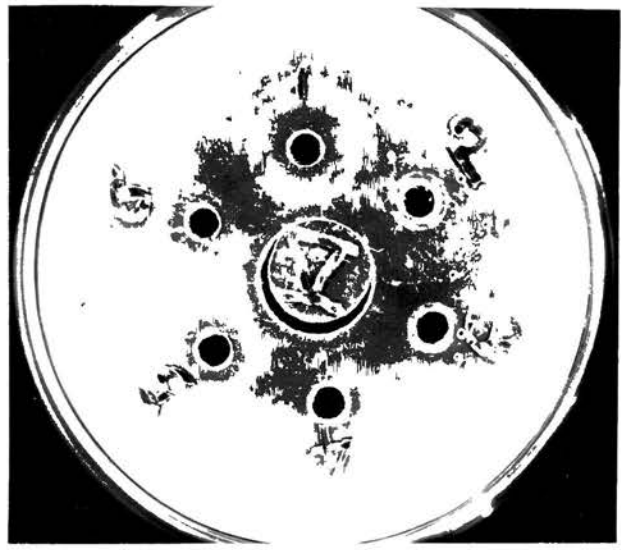

Fig. 21a Quantitative agar gel diffusion precipitation with fraction 5 (human serum) after incorporating antihuman fraction II gamma globulin - rabbit serum (10%) in agar gel. Circular rings of precipitates are formed around first three wells containing neat and 1:5, 1:10 dilutions of the fraction.

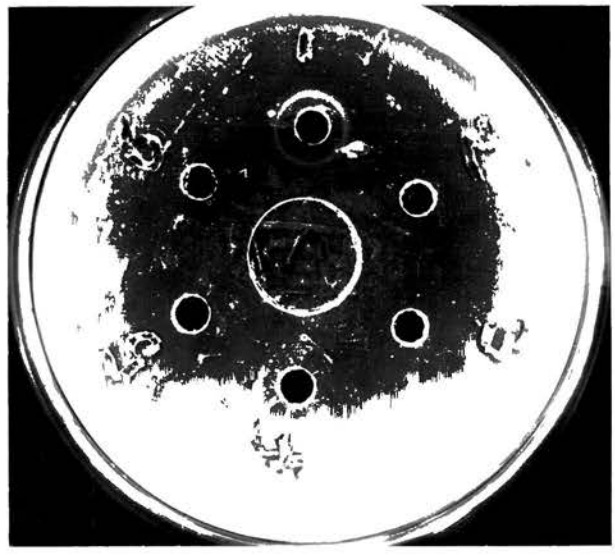

Fig. 21b Quantitative agar gel diffusion precipitation with fraction 7 (human serum) after incorporating antihuman fraction II gamma globulin - rabbit serum (10%) in agar gel. Circular ring of precipitates is formed around the first well only containing the neat fraction.

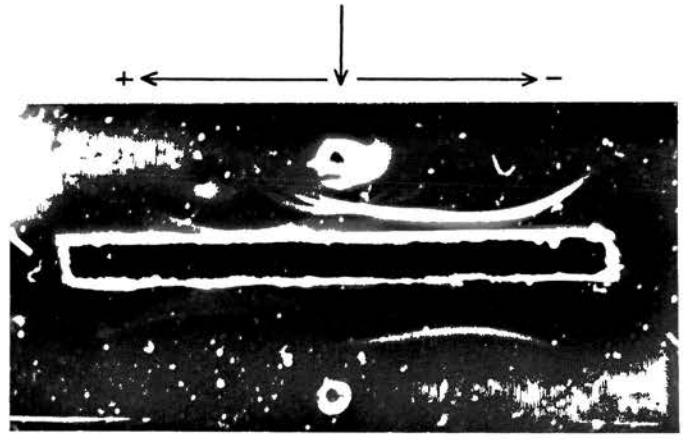

Fig. 22 Agar gel electrophoresis serologic reactions (immuno-electrophoresis) with human sera (in the wells) and antihuman fraction II gamma globulin - rabbit serum (in the gutter). Precipitates are well marked in the region of gamma globulin.

From the results of the quantitative gel diffusion tests (Fig.21a, b) it appears that fraction 5 contains more antigen (gamma globulin) than fraction 7. In contrast the serologic activity was greater in fraction 7 than in fraction 5 (Fig. 24 a, d). This cannot be due to the gamma globulin contaminant of these fractions as fraction 5 is less serologically active but contains more of this contaminant.

Attempts were also successful to remove the remaining trace of gamma globulin from fractions 5 and 7 by repetition of the same process of fractionation (Fig.23). Serologic activities of these fractions were retained in comparison with negative results with the removed gamma globulin fraction (Fig. 24). As the reactivity of such fractions were weak, Wassermann reaction (Price, 1948, 50) was not suitable to detect such activity. So a sensitive method of complement fixation test was carried out in M. R. C. perspex plate using the same antigen (titre 1:250), C. F. T. buffer diluent, 2 x 50% M. H. D. complement and a four drops technique.

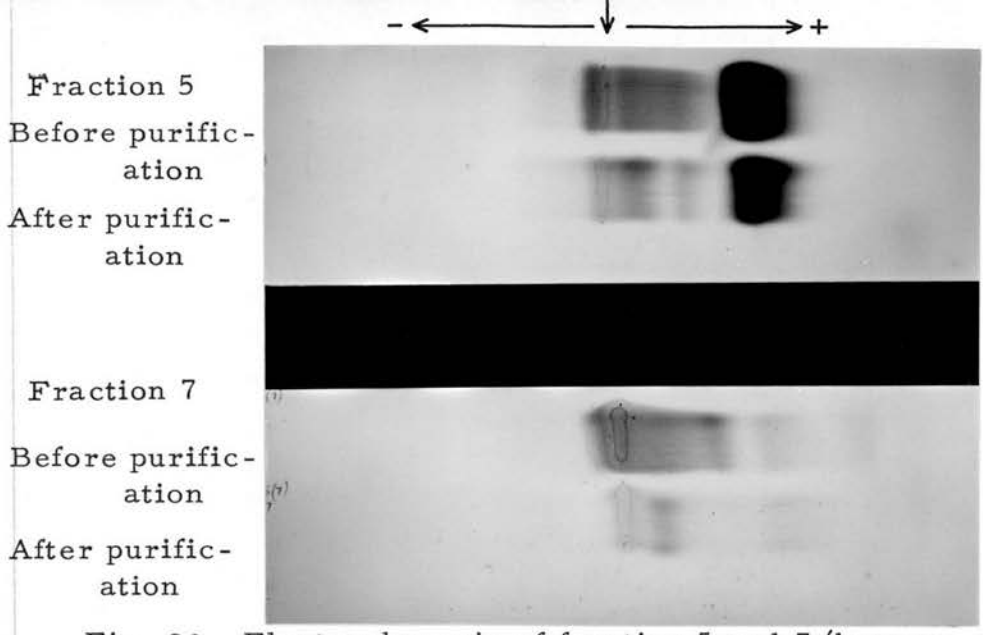

Fig. 23 Electrophoresis of fraction 5 and 7 (human serum) on cellulose acetate paper before and after purification by repetition of fractionation through exchanger column.

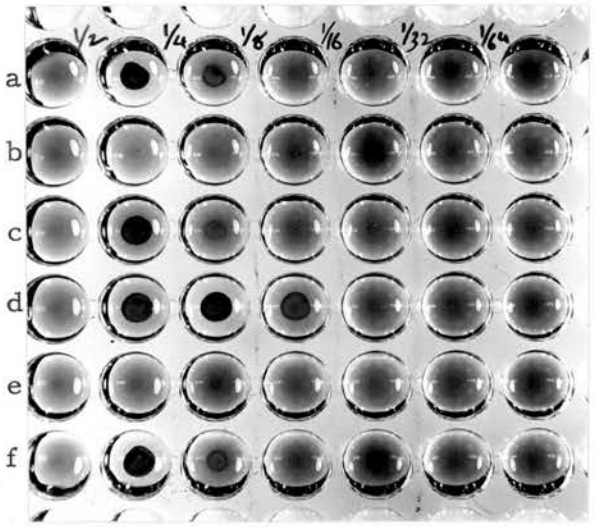

Fig. 24 Complement fixation tests showing the titres of serologic reactions of fraction 5 and 7 (human syphilitic serum) before and after purification and the eluted gamma globulin contaminants.

a. Fraction 5 before purification
b. Eluted gamma globulin contaminant in fraction 5.
c. Fraction 5 after purification.
d. Fraction 7 before purification.
e. Eluted gamma globulin contaminant in fraction 7.
f. Fraction 7 after purification.

Chapter VII

MATERIALS

Serologic reactivities of the different serum protein fractions, after DEAE-Sephadex column chromatography were studied on the samples of sera collected from the following sources.

1. Experimental rabbit syphilis

The amounts of sera left over after study of sensitivities by the different serologic tests in experimental rabbit syphilis (Part I) were used for the fractionation to determine the serologic reactivities of individual fractions (fraction 1, 3, 5 and 7). Sera from all the 9 rabbits were fractionated before they were injected with the treponemes. After infection the sera from the treated group (5 rabbits) were fractionated at a biweekly interval for the first 20 weeks, thereafter at a four-weekly interval until the 40th week of infection. In the untreated group (4 rabbits) similar samples of sera were fractionated starting from the 14th week of infection as it was presumed that the fractions will react similarly as in the treated group in their pre-treatment period.

2. Human sera

(a) Normal healthy adults:

Four sera from normal healthy blood donors were col-

lected from the blood transfusion service, Royal Infirmary, Edinburgh and the serologic reactivities of their protein fractions were studied to compare the same from the diseased individuals.

(b) Natural infection:

Serum fractions were also tested for their serologic reactivities from cases of human syphilis at the different stages. The sera were obtained from the Department of Venereal Disease, Royal Infirmary, Edinburgh. The different stages of the diesease were judged from their clinical manifestations.

The number of sera fractionated for the test of serologic reactivity from different stages of syphilis were as follows:

Primary	2
Secondary	2
Tertiary (late)	2
Latent	4
Congenital	1
TOTAL	11

(c) Biologically false positive sera:

Thirteen fresh samples of sera were available from the biologically false positive reactors detected during the

study of specificity of the serologic tests. These sera were fractionated by the same method of column chromatography and their reactivitieswere tested by the different serologic techniques.

Chapter VIII

RESULTS

Serologic reactivities of the different serum protein fractions

Serum samples were fractionated into four different fractions (Fractions, 1, 3, 5, 7) by DEAE - Sephadex column chromatography and serologic reactivity of each fraction was tested with the Wassermann reaction (WR - Price's), Kahn standard test (KT), VDRL slide flocculation test (VDRL), Reiters protein complement fixation test (RPCF), Treponema pallidum immobilisation test (TPI), and Fluorescent treponemal antibody test (FTA). The results of the serologic reactivities in different fractions of sera collected from experimental rabbit syphilis, different stages of human infection, normal healthy adults and biologically false positive reactors are found to be as follows:

1. Experimental rabbit syphilis:

(a) Treated Group (5 rabbits) - The results of the reactivities of the 6 serologic tests (WR, KT, VDRL, RPCF, TPI, FTA) carried out on each protein fraction (Fractions 1, 3, 5 and 7) of the sera collected from each rabbit of this group at a 2-weekly interval for 20 weeks and then at a 4-weekly interval till the 40th week, are given in 5 different figures (Fig. 25 - B1, Fig. 26 - B2, Fig. 27 - B4, Fig. 28-B5

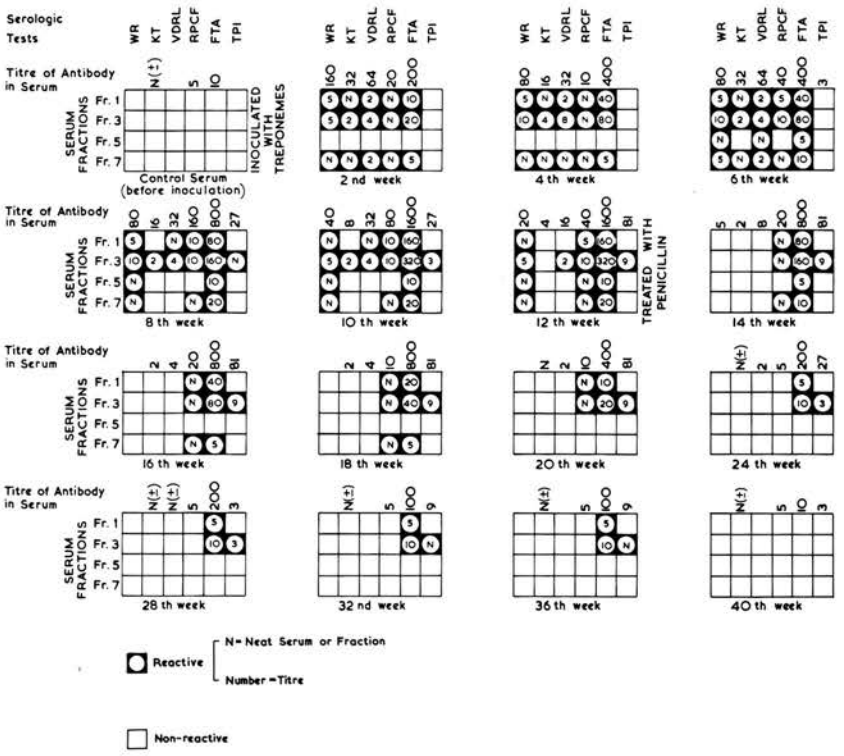

Fig. 25 Titres of the various serologic tests of the sera and their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B1 (treated group).

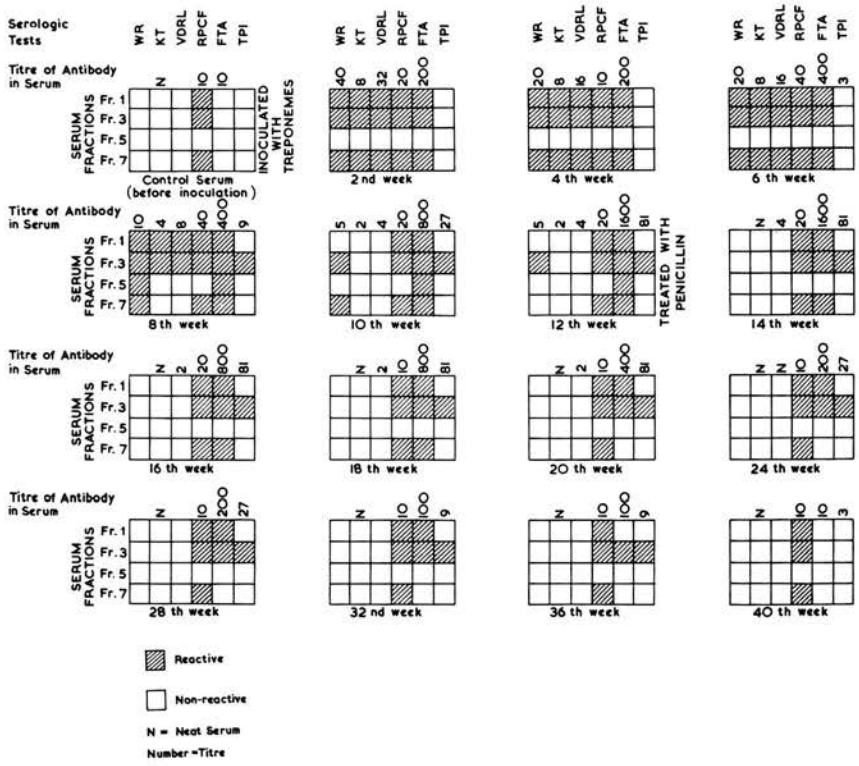

Fig. 26 Titres of the various serologic tests of the sera and the reactivities of their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B2 (treated group).

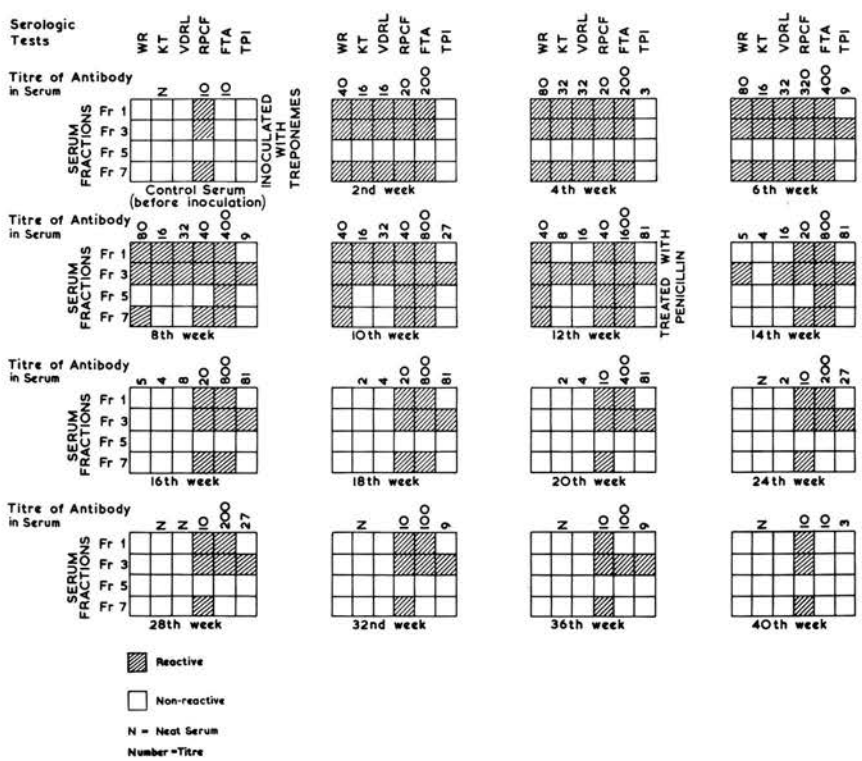

Fig. 27 Titres of the various serologic tests of the sera and the reactivities of their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B4 (treated group).

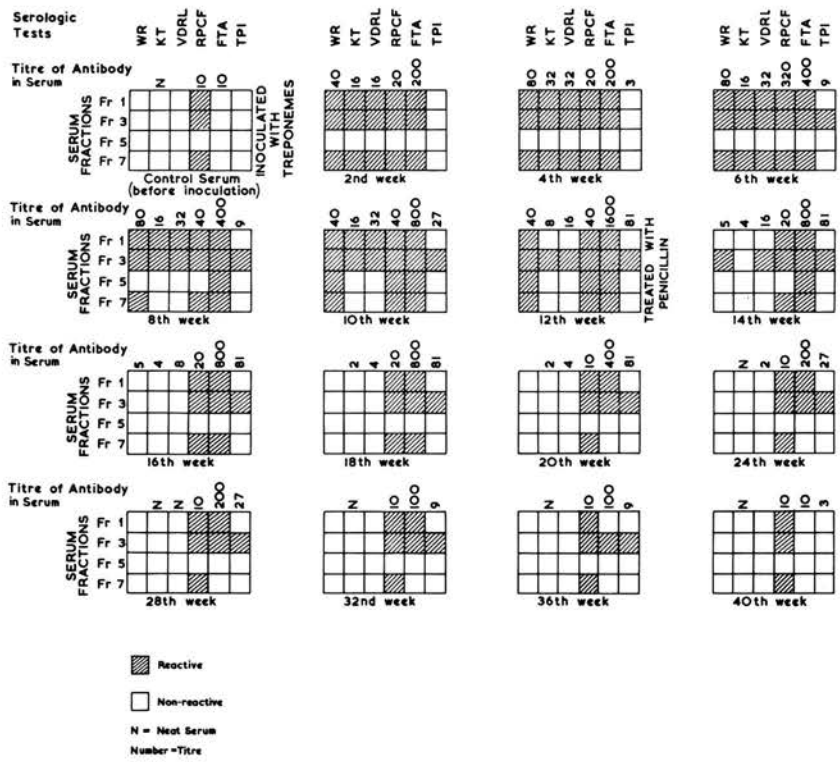

Fig. 28 Titres of the various serologic tests of the sera and the reactivities of their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B5 (treated group).

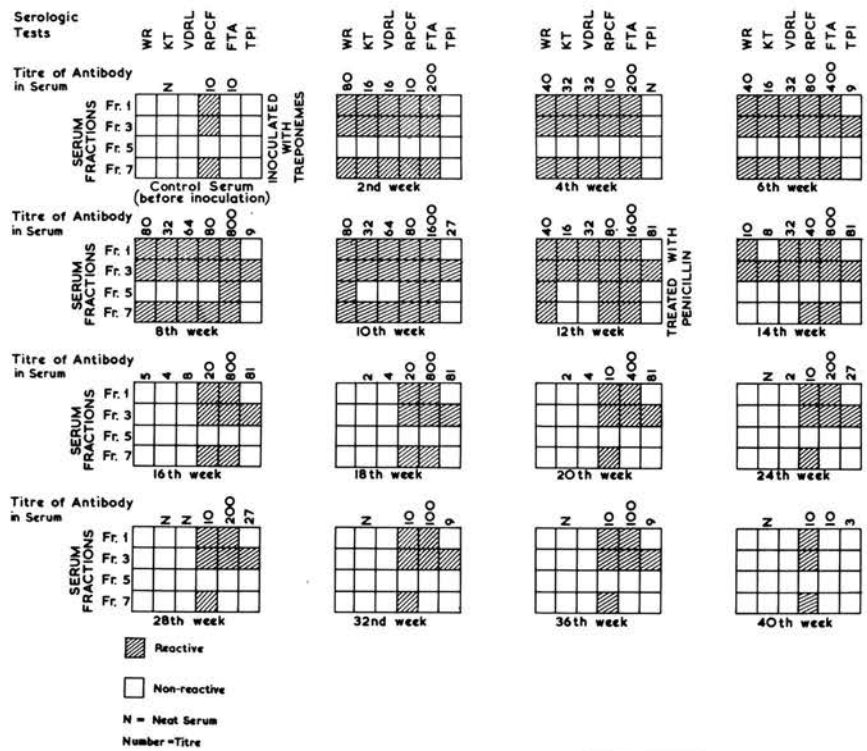

Fig. 29 Titres of the various serologic tests of the sera and the reactivities of their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B6 (treated group).

Fig. 29 - B6) along with the titres of the same sera. The serologic titre of each fraction has been shown only in rabbit No. B1 (Fig. 25) throughout the period of investigation.

Except in rabbit No. B1 (Fig. 25) all the rabbits in this group showed reactivities in fractions 1, 3, and 7 of the pre-infection samples of serum with the RPCF test as this test showed a non-specific reaction with these sera at 1:10 dilution.

On the second week following infection, all the rabbits in this group showed positive reactions in fractions 1, 3 and 7 with all the serologic tests except the TPI.

As the disease progressed fraction 1 showed a gradual increase of titre with the FTA test but a diminution or disappearance of such reactivity was found with the WR, KT, VDRL, RPCF tests even before treatment was started. This finding was associated with the progressive rsie of FTA titre and a fall of titre with the other tests in the serum samples (Fig. 25).

Fraction 3 showed the reactivity at a higher titre (Fig. 25) than that of fraction 1 with all the tests throughout the pre-treatment period and showed its reactivity with all the tests even after disappearance of such reaction with KT and VDRL tests in fraction 1. Reactivity with KT disappeared in fraction

3 even before treatment was started (Fig. 25). The TPI test showed reactivity only with fraction 3 starting on the 6th week in all the rabbits except in rabbit No. B1 (Fig. 25) where the reactivity appeared on the 8th week following infection. The TPI reactivity appeared in this serum fraction from all the rabbits 2 - 4 weeks after the test showed a positive result with the sera as the manifestation of serologic reactivity in this fraction is found to be related to the antibody titre of the serum (Fig. 25).

The reactivity in fraction 5 appeared at a low titre on the 6th week in rabbit No. B1 (Fig. 25) but on the 8th week in other rabbits. This reactivity of fraction 5 is more common with the FTA, RPCF and WR tests although the VDRL test became positive with this fraction in rabbit No. B1 (Fig. 25). The reactivity in fraction 5 is not found to be associated with the increase of titre in the sera in the case of the WR and RPCF tests although such associations are found with the FTA and VDRL tests.

Fraction 7 maintained its reactivity throughout the pre-treatment period with WR, RPCF and FTA tests. VDRL and KT became negative in all the rabbits on the 8th week following infection with the exception of rabbit No. B6 (Fig. 29) where such reactivity disappeared on the 12th week.

In the post treatment period fraction 1 showed disappearance of the reactivities with the non-treponemal tests (WR, VDRL, KT) from the 14th week of infection except in rabbit No. B6 (Fig. 29) where this fraction was positive only with the WR. This fall of reactivity in fraction 1 is found to be associated with the fall of the serum titre. The treponemal test with the FTA maintained reactivity in this fraction till the 36th week in rabbit No. B1 (Fig. 25) and B6 (Fig. 29) whereas it disappeared on the 32nd week in other rabbits. RPCF maintained a positive reactivity till the 20th week in rabbit No. B1 (Fig. 25) but in other rabbits this reactivity cannot be judged due to its non-specific reaction in the control fractions even before infection. This gradual loss ofreactivity with the FTA and RPCF tests are also associated with simultaneous fall in serum titre.

Fraction 3 also showed the same pattern of disappearance of reactivity as was found in fraction 1 after institution of therapy. The disappearance of reactivity in this fraction 3, is noticed mainly with the non-treponemal tests within 2 - 4 weeks after therapy in all the rabbits of this group. The reactivity with the FTA test was retained in all the rabbits till the 36th week. The TPI test showed similar reactivity till the 36th week and disappeared from this fraction along with

the FTA test on the 40th week in all the rabbits when the serum titre was lowered to 1:3. RPCF reactivity disappeared from the fraction 3 on the 24th week in rabbit No. B1 (Fig. 25) though in other rabbits this observation cannot be judged due to the presence of such reactivity in the control fraction. The overall titre of fraction 3 was found to be higher than fraction 1 in all the sera as shown in Fig. 25.

Fraction 5 showed reactivity only with the FTA test on the 14th week in rabbits No. B1, B4, and B5 (Fig. 25, 27, 28) and disappeared completely thereafter.

Fraction 7 in the post-treatment period manifested its reactivity with the RPCF and FTA test. The former reactivity disappeared completely on the 20th week from the rabbit No. B1 (Fig. 25) but in other rabbits it retained the same low activity as was present in the control fractions. The FTA test showed a similar disappearance of reactivity to the RPCF test from the 20th week in all the rabbits.

(b) Untreated group (4 rabbits) - The results of the serologic reactivities of 6 tests (WR, KT, VDRL, RPCF, FTA, TPI) carried out on each protein fraction of the sera collected from each rabbit from the 14th week of infection at a 2-weekly interval for 20 weeks and then at a 4-weekly interval till the 40th week, are given in 4 different figures (Fig. 30 - B7,

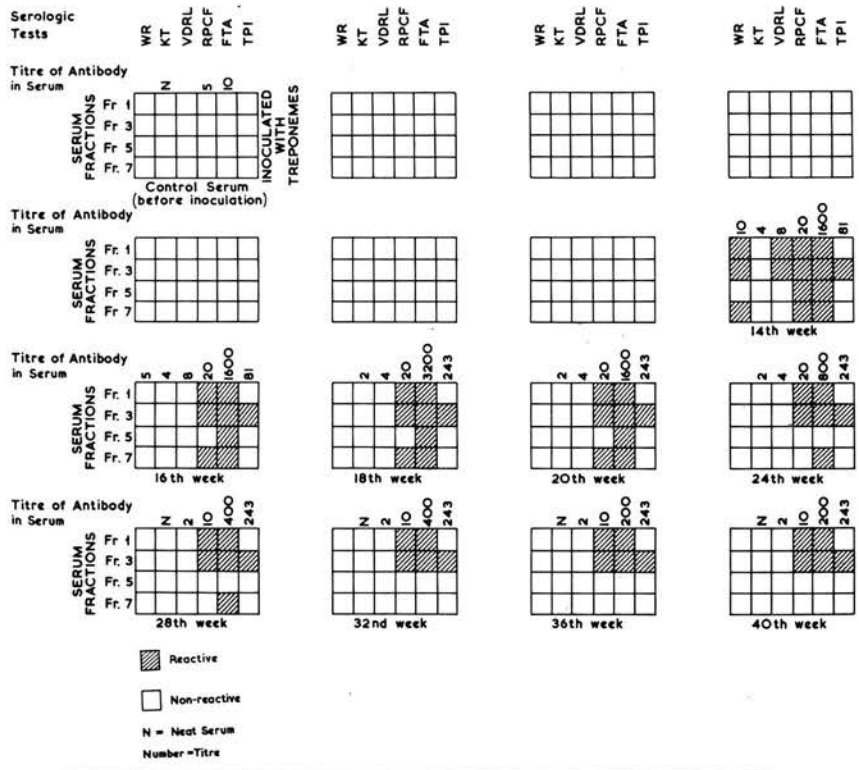

Fig. 30 Titres of the various serologic tests of the sera and the reactivities of their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B7 (untreated group).

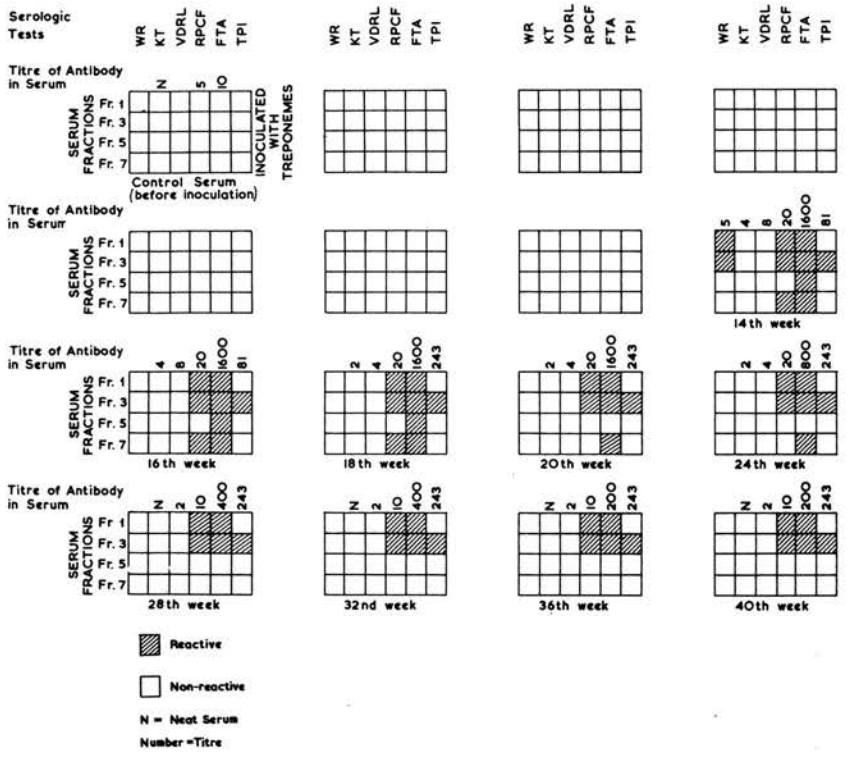

Fig. 31 Titres of the various serologic tests of the sera and the reactivities of their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B8 (untreated group).

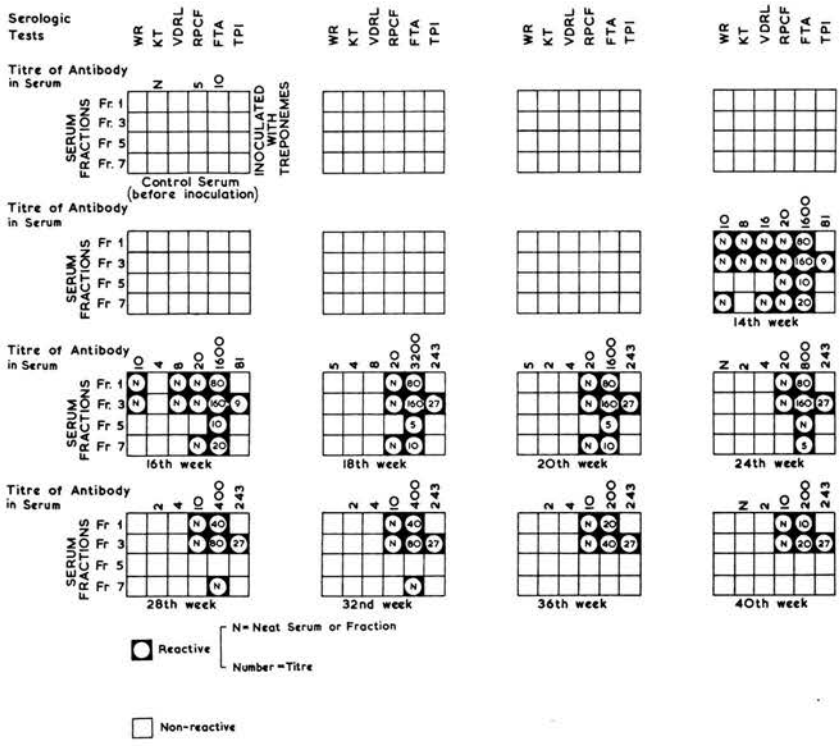

Fig. 32 Titres of the various serologic tests of the sera and their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B9 (untreated group).

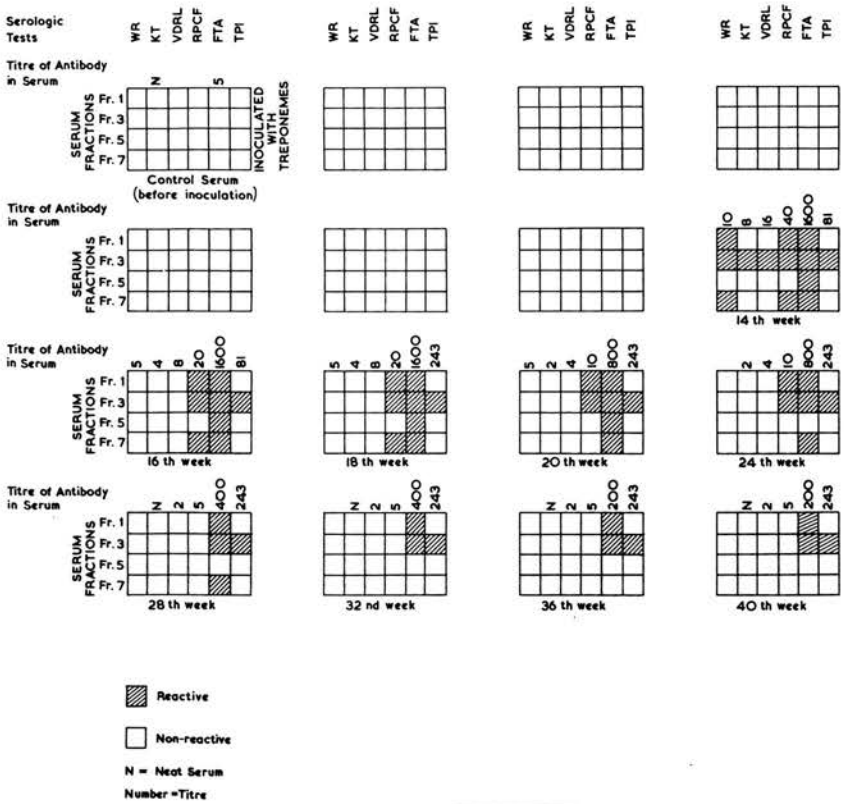

Fig. 33 Titres of the various serologic tests of the sera and the reactivities of their fractions at a periodic interval after intratesticular inoculation of Nichols strain treponemes in rabbit No. B10 (untreated group).

Fig. 31 - B8, Fig. 32 - B9, Fig. 33 - B10) along with the serologic titre of the same serum sample. The titre of each fraction has been shown only in one rabbit of this group (Fig. 32) throughout the period of investigation.

In this group the protein fractions of the control sera did not show any reactivity with any of the serologic tests as the titre of the non-specific reactivity in the whole sera with KT, RPCF and FTA tests are low.

On the 14th week of infection, fraction 1 showed serologic reactivity with the WR, KT, VDRL, RPCF and FTA tests in rabbit no. B9 (Fig. 32). Rabbit No. B7 (Fig. 30), behaved similarly but without any activity with KT. Other two rabbits reacted only with the WR, RPCF and FTA tests.

Fraction 3 showed reactivity with all the 6 tests including the TPI in rabbit No. B9 (Fig. 32) and B10 (Fig. 33) but disappearance of such reactivity with KT and VDRL in rabbit No. B8 (Fig. 31) and with KT only in rabbit no. B7 (Fig. 30) was noted. The titre of this fraction was higher than that of other fractions as was manifested by the FTA test (Fig. 32).

Reactivity in fraction 5 persisted with the RPCF and FTA tests in rabbit no. B9 (Fig. 32) and B7 (Fig. 30), whereas only the FTA test showed such reactivity in other two rabbits.

Fraction 7 maintained the reactivity with WR, VDRL, RPCF and FTA tests in rabbit no. B9 (Fig. 32), with WR, RPCF and FTA tests in rabbit no. B7 (Fig. 30) and B10 (Fig. 33) whereas rabbit no. B8 (Fig. 31) showed such activity with RPCF and FTA tests only.

As the disease progressed, the reactivity with the non-treponemal tests disappeared from all the fractions in all the rabbits on the 16th week of infection except in rabbit No. B9 (Fig. 32) where the disappearance was complete on the 18th week. With the treponemal tests fraction 1 showed positive results with the RPCF and FTA tests throughout the whole period except in rabbit no. B10 (Fig. 27) where the RPCF reactivity disappeared on the 28th week. The titre of the FTA test in this fraction became lowered to a marked extent with the progress of the disease as shown in Fig. 32. This fall in titre is related to the fall of serum titre.

Similarly, fraction 3 showed reactivity with all the treponemal tests (RPCF, FTA, TPI) throughout the whole period of observation in all the rabbits except in rabbit No. B10 (Fig. 33), where RPCF reactivity disappeared on the 28th week. The disappearance of RPCF reactivity in rabbit no. B8 (Fig. 31) and B9 (Fig. 32) were noted on the 20th and 28th week respectively.

Fraction 5 showed positive results only with the FTA test in all the rabbits of this group at this stage of the disease but the reactivity persisted in this fraction till the 20th week in rabbit no. B7 (Fig. 30) and B10 (Fig. 33) whereas rabbit no. B8 (Fig. 31) and B9 (Fig. 32) showed positive results till the 18th and 24th weeks respectively.

Fraction 7showed reactivity with the RPCF test until the 20th week in rabbit no. B7 (Fig. 30) and B9 (Fig. 32) whereas in the other two rabbits the reactivity was absent. FTA reactivity persisted in this fraction for a longer period in comparison with the other tests but disappeared in rabbit no. B9 (Fig. 32) on the 36th week, in rabbit no. B7 (Fig. 30) and B10 (Fig. 30) on the 32nd week whereas in rabbit no. B8 (Fig. 31) it disappeared earlier on the 28th week of infection.

2. Human sera:

(a) Normal healthy adults. Table XXII shows the results of the 6 serologic tests (WR, KT, VDRL, RPCF, FTA TPI) with four sera and their fractions (1, 3, 5, 7) collected from normal healthy adults. None of the sera or their fractions showed any reactivity with any of the serologic tests.

(b) Natural infection. Table XXIIIshows the results of

Table XXII showing reactive patterns of serum fractions from healthy blood donors with the different serologic tests.

Sr. no.	serum no.	serologic tests	serum	Serum fractions			
				1	3	5	7
1	N_1	Wr (Price's)	Neg	-	-	-	-
		KT	Neg	-	-	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA-200	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
2	N_2	WR (Price's)	Neg	-	-	-	-
		KT	Neg	-	-	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA:200	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
3	N_3	WR (Price's)	Neg	-	-	-	-
		KT	Neg	-	-	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA:200	Neg	-	-	-	-
4	N_4	WR (Price's)	Neg	-	-	-	-
		KT	Neg	-	-	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA:200	Neg	-	-	-	-
		TPI	Neg	-	-	-	-

Table XXIII showing reactive patterns of serum fractions from different stages of Natural infection (syphilis) with the different serologic tests.

Sr. No.	Serum No.	Stage of disease	Serologic tests	Serum	Serum fractions 1	3	5	7
1	FT6	Primary (D. G. +ve)	WR(Price's)	Pos(10 dils)	+	±	-	±
			KT	Pos(2 dils)	+	-	-	-
			VDRL	Wpos(4 dils)	+	-	-	±
			RPCF	Pos(10dils)	+	±	-	++
			FTA	Pos(800 dils)	+	+	-	++
			TPI	Doubtful ±	-	-	-	-
2	FT7	Primary (D. G. +ve)	WR(Price's)	Pos(5 dils)	+	+	-	-
			KT	Pos(2 dils)	+	-	-	-
			VDRL	Pos(4 dils)	+	-	-	±
			RPCF	Pos(5 dils)	+	±	-	-
			FTA	Pos(400 dils)	+	+	-	+
			TPI	Neg	-	-	-	-
3	S9	Congenital	WR (Price's)	Pos (5 dils)	+	-	-	-
			KT	Pos (2 dils)	+	-	-	-
			VDRL	Pos (4 dils)	+	-	-	-
			RPCF	Wpos(5dils)	+	-	-	-
			FTA	Pos (200dils)	+	-	-	-
			TPI	Pos (9 dils)	+	-	-	-
4	S6	Secondary	WR (Price's)	Pos(20 dils)	+	±	-	+
			KT	Pos (8 dils)	+	-	-	-
			VDRL	Pos(16dils)	+	-	-	+
			RPCF	Pos(40dils)	+	+	-	+
			FTA	Pos(1600dils)	+	+	-	+
			TPI	Pos(81dils)	+	-	-	-
5	S8	Secondary	WR (Price's)	Pos(10dils)	+	±	-	±
			KT	Pos(8 dils)	+	±	-	±
			VDRL	Pos(16dils)	+	±	-	±
			RPCF	Pos(20dils)	+	±	-	±
			FTA	Pos(1600dils)	+	+	-	+
			TPI	Pos(81dils)	+	-	-	-
6	S2	Tertiary(late)	WR (Price's)	Pos (5 dils)	+	-	-	±
			KT	Pos (4 dils)	+	-	-	+
			VDRL	Pos (8 dils)	+	±	-	+
			RPCF	Pos (10dils)	+	±	-	+
			FTA	Pos(800dils)	+	+	+	+
			TPI	Pos(243dils)	+	-	-	-
7	S7	Tertiary(late)	WR (Price's)	Pos (5 dils)	+	-	-	+
			KT	Pos (4 dils)	+	-	-	±
			VDRL	Pos (8 dils)	+	-	-	±
			RPCF	Pos (10dils)	+	+	-	±
			FTA	Pos(800dils)	+	+	+	+
			TPI	Pos(81dils)	+	-	-	-
8	S1	Latent	WR (Price's)	Neg	-	-	-	-
			KT	Pos (4 dils)	+	-	-	-
			VDRL	Pos (8 dils)	+	-	-	-
			RPCF	Pos(10dils)	+	+	-	±
			FTA	Pos(400dils)	+	+	+	+
			TPI	Pos (27dils)	+	-	-	-
9	S3	Latent	WR (Price's)	Wpos(5dils)	+	-	-	+
			KT	Pos (8 dils)	+	-	-	±
			VDRL	Pos (16dils)	+	-	-	+
			RPCF	Pos (10dils)	+	±	±	+
			FTA	Pos(400dils)	+	+	+	+
			TPI	Pos (81dils)	+	-	-	-
10	S4	Latent	WR (Price's)	Pos (5 dils)	±	-	-	±
			KT	Pos (4 dils)	±	-	-	±
			VDRL	Pos(16dils)	±	-	-	±
			RPCF	Pos(20dils)	±	-	-	±
			FTA	Pos(400dils)	+	±	±	+
			TPI	Pos (81dils)	+	-	-	-
11	S5	Latent	WR (Price's)	Pos (20dils)	+	±	±	+
			KT	Pos (8 dils)	+	-	-	±
			VDRL	Pos(32dils)	+	-	±	+
			RPCF	Pos(40dils)	+	+	±	+
			FTA	Pos(1600dils)	+	+	+	+
			TPI	Pos(243dils)	+	-	-	-

the serologic reactions of the same 6 serologic tests with the sera and their fractions obtained from 11 cases of untreated syphilis at the different stages of the disease (primary - 2, secondary - 2, tertirary (late) - 2, latent - 4, congenital - 1).

In the primary cases (serum no. FT6 and FT7) the FTA test showed its reactivity at a higher titre in comparison with other tests whereas the TPI test was negative in one serum (FT7) and in the other (FT6) it showed a doubtful result. Fraction 1 from both the sera reacted with all the tests except the TPI. Fraction 3 showed positive results with the WR, RPCF and FTA tests in both the sera. Fraction 5 of these sera remained non-reactive with all the tests whereas fraction 7 showed reactivity with the RPCF and FTA tests in both the sera in association with a weak reactivity of WR and VDRL with the serum no. FT6.

The sera from secondary syphilis (S6 and S8) reacted at a high titre with all the serologic tests. Reactivity with all the tests was also manifested in fraction 1 from these sera. Fraction 3 showed positive results with all the tests except the TPI in serum no. S8 whereas in serum no. S7, the reactivity in this fraction was restricted to the WR, RPCF and FTA tests. Fraction 5 from both the sera was negative with all the tests. Fraction 7 showed positive results with

all the tests except the TPI in serum No. S8, whereas this fraction from serum no. S6 reacted with the WR, VDRL, RPCF and FTA tests.

The titres of the two sera (S2 and S7) collected from the tertiary stages of syphilis were higher with the FTA and TPI in comparison with the other tests. Fractions from both the sera showed similar reactivity with the different serologic tests. Fraction 1 showed positive results with all the tests but fraction 3 reacted only with the RPCF and FTA tests. The serologic reactivity was also manifested in fraction 5 showing a positive result only with the FTA test. Fraction 7 showed the similar results as fraction 1 manifesting its reactivities with all the tests.

Four sera (S1, S3, S4, S5) from latent cases of syphilis showed a moderate titre with all other serologic tests except the WR which was negative with the serum no. S1. Fraction 1 from all these sera showed reactivity with all the tests except the WR which showed negative result with this fraction from serum no. S1 as this test showed a negative result with the serum. The reactivity in fraction 3 was manifested with the RPCF and FTA tests in two sera (S1 and S3) whereas this fraction from serum no. S4 showed reactivity only with the FTA test and from serum no. S5 reacted with

the WR, RPCF and FTA tests. Reactivity was also noticed with the FTA test in fraction 5 from allthese sera although the RPCF test showed a positive reaction in serum no. S3 and S5 whereas the WR and VDRL tests were also positive with this fraction from serum no. S5. Fraction 7 showed positive results with WR, KT, VDRL, RPCF, and FTA tests in all these sera except serum no. S1 where no such reactivity was found with KT and VDRL tests.

The serum (S9) from the congenital case of syphilis showed a poor serologic reactivity with all the tests except the FTA which was positive at 1:200 dilution. The reactivity of the fractions from this serum was restricted only to fraction 1 with all the serologic tests.

(c) Biologically false positive sera: Table XXIV showed the reactivity of 6 serologic tests carried out on 13 sera and their fractions obtained from biologically false positive reactors. All these sera showed poor reactivities only with the 3 non-treponemal tests (WR, KT, VDRL) either becoming positive with all the tests at the same time (serum no. 41, 115, 144, 264) or with the two tests together (serum no. 104, 109, 116, 131, 181, 202, 262) or with a single test (serum no. 72, 213). Only the fraction 1 showed the serologic activity in 9 out of 13 sera (serum no. 41, 72, 104, 109, 115, 116, 131,

Table XXIV showing reactive patterns of serum fractions from "Biologically false positive" cases with the different serologic tests.

Sr. no.	Serum no.	Serologic tests	Serum	Serum fractions 1	3	5	7
1	41	W. R. (Price's)	Pos (5 dils)	+	-	-	-
		KT	Pos (2 dils)	+	-	-	-
		VDRL	Pos (4 dils)	+	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
2	72	WR (Price's)	Neg	-	-	-	-
		KT	Pos (2 dils)	+	-	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
3	104	WR (Price's)	Neg	-	-	-	-
		KT	Pos (2 dils)	±	-	-	-
		VDRL	Pos (4 dils)	+	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
4	109	WR (Price's)	Neg	-	-	-	-
		KT	Pos (2 dils)	+	-	-	-
		VDRL	Pos (4 dils)	+	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
5	115	WR (Price's)	Pos (5 dils)	+	-	-	-
		KT	Pos (2 dils)	±	-	-	-
		VDRL	Pos (4 dils)	+	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
6	116	WR (Price's)	Pos (5 dils)	+	-	-	-
		KT	Pos (2 dils)	+	-	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-

Table XXIV continued:

Sr. no.	Serum no.	Serologic tests	Serum	Serum fractions 1	3	5	7
7	131	WR (Price's)	Pos (5 dils)	+	-	-	-
		KT	Pos (2 dils)	+	-	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
8	144	WR (Price's)	W pos (5 dils)	±	-	-	-
		KT	Pos (N)	-	-	-	-
		VDRL	W pos (2 dils)	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
9	181	WR (Price's)	Neg	-	-	-	-
		KT	Pos (2 dils)	+	±	-	-
		VDRL	W pos (4 dils)	+	±	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
10	202	WR (Price's)	Neg	-	-	-	-
		KT	Pos (2 dils)	-	±	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
11	213	WR (Price's)	Neg	-	-	-	-
		KT	Pos (2 dils)	-	±	-	-
		VDRL	Neg	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
12	262	WR (Price's)	Neg	-	-	-	-
		KT	Pos (N)	-	-	-	-
		VDRL	W pos (2 dils)	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-
13	264	WR (Price's)	W pos (5 dils)	±	-	-	-
		KT	Pos (N)	-	-	-	-
		VDRL	W pos (2 dils)	-	-	-	-
		RPCF	Neg	-	-	-	-
		FTA	Neg	-	-	-	-
		TPI	Neg	-	-	-	-

144, 264) whereas the reactivity was restricted to fraction 3 only in one serum (no. 213). Both the fractions 1 and 3 showed the reactivity only in one serum (no. 181) although reactivity was completely absent from all the fractions in 2 sera (no. 202, 262). The manifestation of the reactivity with the different tests in these fractions are found to be related to the titre of such tests in the serum before fractionation, as the reactivities are absent in all these fractions (serum no. 202, 262) when the serum showed a weak positive reaction at 1:2 dilution or was reactive only with the neat serum. It is important to note that fractions 5 and 7 did not show any reactivity with any of the serologic tests in this group of biologically false positive sera.

To summarise, the reactivities of the different serologic tests in serum fractions from the different stages of experimental rabbit syphilis and human infection, biologically false positive reactors and normal healthy adults are found to be as follows:

1. Experimental rabbit syphilis: The manifestation of serologic reactivities was noted with the WR, KT, VDRL, RPCF, and FTA tests in fractions 1, 3 and 7 from the beginning of the infection in the rabbit but the TPI test showed negative results with all the fractions at this earlier stage of infection.

With the advancement of the disease process the serologic reactivity of all the tests (except the TPI) migrate to fraction 5 and the appearance of a positive result in this fraction is found to be related to the relative sensitivity of the tests. The TPI reactivity appeared only in fraction 3 at this advanced stage of the disease and retained its reactivity in this fraction throughout this stage.

In the late stage of the disease in untreated rabbits, the gradual disappearance of reactivity from fractions 5, 7, 1, 3 in that order, was noticed with the less sensitive non-treponemal tests (KT, VDRL, WR) whereas the reactivity was maintained in fraction 1 and 3 with the more sensitive and specific tests like RPCF and FTA of the newer treponemal group till the end of 48 weeks although they showed negative results earlier with fraction 5 than fraction 7. The TPI test showed positive results only with fraction 3 in this late stage of the disease.

In the late stages of treated rabbit syphilis all the tests belonging to non-treponemal and newer treponemal groups behaved similarly in the disappearance of their reactivities from fractions 5, 7, 1, and 3 in that order, but the disappearance of such reactions with the non-treponemal (KT, VDRL, WR) group was noticed earlier than with the newer treponemal group of

tests (FTA, RPCF). This corresponded with the lower antibody titres in the whole sera detected by the former group of tests in comparison with that of the latter group, which subsequently determined the appearance of serologic reactivity in the serum fractions. The TPI test showed positive results only with fraction 3 throughout this stage and its reactivity disappeared from this fraction when the titre of the whole serum was found to be poor.

2. Human syphilis: Fractions 1, 3 and 7 from human sera in the early stage of the disease reacted similarly with all the serologic tests as was found in the early stage of rabbit syphilis but the appearance of such reactivity was related to the sensitive nature of the serologic tests.

In the secondary stage of human infection, serologic reactivity was still restricted in the same serum fractions as in the early stage without any tendency of migration to fraction 5 as was found in the advanced stage of experimental rabbit syphilis. But the TPI reactivity was found to be restricted to fraction 1 in contrast to rabbit serum where such reaction was noticed in fraction 3.

In the late stage of untreated human syphilis, the migration of serologic reactivities from fractions 1, 3, 7 to frac-

tion 5 was noticed in striking contrast to rabbit syphilis where such migration was noticed earlier in the course of the disease with the disappearance of the serologic reactivity from fractions 5 and 7 even without treatment. The TPI test still maintained its activity in fraction 1 in contrast to the late stage of rabbit syphilis where such reactivity was manifested only in fraction 3.

In congenital syphilis, all the serologic tests including the TPI showed positive results in fraction 1.

3. Normal human adults: All the serologic tests showed negative results with all the serum fractions collected from normal human adults.

4. Biologically false positive reactors: In contrast to the findings of serologic reactivity in different fractions in human and rabbit syphilis, the biologically false positive reactors showed reactivity only in fractions 1 and 3 and only the non-specific tests like the WR, KT and VDRL manifested such reactions. The appearance of reactivity in these fractions was also related to the whole serum and the relative sensitivity of these tests.

Chapter IX
COMPARATIVE ANALYSIS OF SEROREACTIVITY IN SERUM PROTEIN FRACTIONS FROM NORMAL, SYPHILITIC AND BIOLOGICALLY FALSE POSITIVE SERA

The serologic reactivities of the different globulin fractions of serum have been studied in different stages of experimental rabbit and human syphilis with the WR, KT, VDRL, TPI, RPCF and FTA tests to find out the differences in their reactivities with the non-treponemal (WR, KT, VDRL) and treponemal (TPI, RPCF, FTA) groups of tests at different stages of the disease.

Along with this study of serum protein fractions in experimental rabbit syphilis and in human syphilis, a similar study of serologic reactivity with the different globulin fractions from biologically false positive and normal human sera was also carried out. Such a study of comparative reactivity was made with a view to finding an explanation for the reaction in biologically false positive sera according to the serologic activity of their globulin fractions, if the reactivity pattern in these fractions differs from that of human syphilitic serum. If that were so, the simpler non-specific serologic tests like the WR, KT and VDRL could be used for the identification of biologically false positive sera from their reactivities with the s eparated fractions. The other purpose of this comparative study was to find out the

nature of the reagin present in the globulin fraction, as qualitative or quantitative changes or a change instructural configuration (as found in antibody molecules) in the normal globulin fraction, might be responsible for such abnormal reactions like the reagin activity. The classical liver function flocculation tests and colloidal gold precipitation tests of C. S. F. support this idea of abnormal precipitation reactions due to quantitative changes of albumin and globulin in the reacting fluids tested.

The use of DEAE-Sephadex column chromatography has simplified the procedure of fractionating serum globulin into four components (Fractions 1, 3, 5, 7). When the purity of these fractions is tested by cellulose paper electrophoresis (Fig. 17, 18) and agar gel immunoelectrophoresis (Fig. 19) fraction 1 is found to be pure $gamma_2$ globulin whereas other fractions are found to be mixtures of two globulin components (thus fraction 3 is a mixture of $gamma_1$ and $gamma_2$ globulins; fraction 5 contains $alpha_1$ and $alpha_2$ globulins, whereas fraction 7 is composed of $alpha_2$ and beta globulins). However, the part played by the individual globulin fractions could be ascertained when the fractions 3, 5, and 7 show serologic activities as have been described on page 138 and page 163.

The serologic activities observed in different globulin fractions at different stages of experimental rabbit syphilis and in human infections, are found to be as follows:

1. EARLY STAGE OF SYPHILIS

(1) Experimental rabbit syphilis (0-8 weeks after infection) (Figs. 25, 26, 27, 28, 29)

In the beginning of this early stage of the disease on the second week of infection, WR, KT, VDRL, RPCF and FTA tests showed positive results with fraction 1 ($gamma_2$ globulin), fraction 3 ($gamma_1$ and $gamma_2$ globulins) and fraction 7 (beta globulin). The TPI test did not react with any of these fractions as the test was negative with the original serum before fractionation. As the disease progressed, the serologic reactivity appeared in fraction 5 (alpha globulin) starting on the sixth week of infection. The reactivity in this fraction was manifested first with the more sensitive tests like FTA, WR and RPCF corresponding with the high titres in the original serum before fractionation in comparison with the KT and VDRL tests. The TPI activity also appeared in fraction 3 ($gamma_1$ and $gamma_2$ globulins) on the sixth week of infection at a time when this test showed a positive result with the serum at a significantly high level. Thus with the onset of the disease process in the rabbit, seroreactivity appears in $gamma_1$, $gamma_2$ and beta globulins with the WR, KT, VDRL, RPCF and FTA tests and as the disease progresses, this reactivity spreads to alpha globulin along with the appearance of TPI activity only with the $gamma_1$ and $gamma_2$ globulins (fraction 3).

(2) Early primary human syphilis (Table XXIII)

In two sera from primary syphilis, seroreactivities were found with the WR, KT, VDRL, RPCF and FTA tests in fraction 1 (Gamma$_2$ globulin) whereas fraction 3 (mainly gamma$_1$ globulin) showed positive results only with the more sensitive tests like the WR, RPCF and FTA tests. Although the reactivity of fraction 7 (beta globulin) varied in two sera, this fraction was positive with sensitive tests like the FTA and RPCF. The TPI did not react with any of these fractions as the test was negative in one serum and showed a doubtful reaction in another.

Thus, in this early stage of human infection, the WR, KT, VDRL, RPCF and FTA tests manifest their reactivity mainly in gamma2 globulin although such reactivities are also found in gamma1 (macroglobulin) and beta globulins with the sensitive tests like FTA, RPCF and WR.

This difference in manifestation of reactivities in gamma$_1$ and gamma$_2$ globulins in the early stage of human and rabbit syphilitic sera may be related to the finding that in the case of rabbit serum, gamma$_2$ globulin splits into two fractions during the fractionation procedure, the minor portion migrates to fraction 1 as a distinct component and the major one to fraction

3 along with gamma$_1$ globulin, whereas in human serum, gamma$_2$ globulin is mostly found in fraction 1 although a trace is present in fraction 3 along with gamma$_1$ (macroglobulin) globulin.

II. ADVANCED STAGE OF SYPHILIS

(1) Experimental rabbit syphilis (8-12 weeks after infection)
(Figs. 25, 26, 27, 28, 29)

As no secondary manifestations were found in rabbits, this stage was considered when the antibody titre of the sera was highest with most of the serologic tests during the period of 48 weeks of observation. At this stage of the disease although the serologic activities were found in all the globulin fractions of the sera, the manifestation of reactivities in these fractions was related to the degree of sensitivity of the tests. Thus the FTA, RPCF and WR showed positive results with all the globulin fractions throughout this stage whereas the serologic reactivity with the KT and VDRL tests disappeared from alpha, beta and gamma$_2$ globulins in successive order as the disease progressed and KT showed negative results with all the fractions even before the treatment was started. This disappearance of serologic reactivity in the globulin fractions with the different tests is related mainly to the serum titre. and thus reflects the sensitivity of the test.

The TPI test showed positive results only with fraction 3 which consists of gamma$_1$ and gamma$_2$ globulins.

Thus in the advanced stage of rabbit syphilis, gamma1 and gamma$_2$, beta and alpha globulins of serum showed reactivities with the WR, KT, VDRL, RPCF and FTA tests depending upon the sensitivity of these tests whereas the TPI test showed a positive result only with fraction 3 which is composed of gamma$_1$ and gamma$_2$ globulins.

(2) Secondary Stage of Human Syphilis (Table XXIII)

In this florid stage of secondary syphilis, the serologic reactivities were restricted to gamma$_1$, gamma$_2$ and beta globulin fractions of the serum. The manifestations of seroreactivity of these different globulin fractions depend upon the sensitivity of serologic tests except in the case of the TPI test which was positive only with gamma$_2$ globulin. No serologic activity was found in alpha globulin fraction with any of the serologic tests at this stage of the disease in striking contrast to the positive finding in the advanced stage of experimental rabbit syphilis.

Thus, in this secondary stage of human infection, the serologic reactivities of the WR KT, VDRL, RPCF and FTA tests are restricted to gamma$_1$, gamma$_2$ and beta globulins of sera and the manifestations of their reactivities in these

fractions are related to the sensitivity of the test concerned, whereas the TPI test showed a positive result only with gamma$_2$ globulin. The peculiar behaviour of the TPI test in manifesting reactivity with gamma$_1$ and gamma$_2$ globulins of rabbit serum and gamma$_2$ globulin of human serum, may be explained from the following findings:

Although the reactivity of the TPI test in the rabbit serum was found to be restricted in fraction 3 (gamma$_1$ and gamma$_2$ globulins), a doubtful reaction was also noticed in fraction 1 (gamma$_2$ globulin) which was not recorded throughout the investigation. This splitting of the TPI reactivity in these two fractions indicates that the gamma$_2$ globulin was responsible for such reactivity. The complete absence of TPI reactivity in fraction 3 (mainly gamma$_1$ globulin) from human serum also supports the view that the TPI reactivity of fraction 3 in rabbit serum is probably due to gamma$_2$ globulin activity only. The TPI antibody is likely to be more concentrated in gamma$_2$ globulin of fraction 3 than that of fraction 1 in rabbit serum.

III. LATE STAGE OF SYPHILIS

(1) Experimental rabbit syphilis (12-48 weeks after infection)

(a) Treated group of rabbits (Figs. 25, 26, 27, 28, 29) - Although all the globulin fractions of the serum showed positive

results with most of the serologic tests (depending upon sensitivity of these tests) before the treatment was started, the reactivities in these fractions begin to disappear earlier - just after the introduction of treatment in comparison with the serum fractions from the untreated group of rabbits. The reactivities of KT, VDRL, WR, RPCF and FTA tests disappeared successively in this order: early from the alpha globulin, followed by beta, $gamma_2$ and $gamma_1$ globulins. Thus KT becomes negative first in alpha globulin even before treatment was started and was followed by the disappearance of reactivities with the VDRL, WR, RPCF and FTA tests at the different periods after treatment. Similar results were found with beta, $gamma_2$ and $gamma_1$ globulins respectively. Thus the reactivity of the $gamma_1$ globulin with the FTA test disappeared at last on the 26th week after treatment. This disappearance of reactivity of the different serologic tests from different globulin fractions was found to be related to the titres of these tests in the sera before fractionation.

The TPI test showed positive results with the $gamma_2$ globulin of fraction 3 throughout this period and the test became negative on the 26th week after treatment when the titre in the serum was low.

(b) Untreated group of rabbits (Figs. 30, 31, 32, 33) - In this late stage of the disease, the untreated group of rabbits showed serologic activity mostly with the WR, KT, VDRL, RPCF and FTA tests in all the globulin fractions on the 14th week of infection, again corresponding with the titre of serologic reactions with the whole serum. As the disease progressed, the reactivities of the less sensitive non-treponemal group of tests (WR, KT, VDRL) began to disappear gradually from all these fractions successively from alpha, beta, $gamma_2$ and $gamma_1$ globulins as the antibody titres in the serum revealed by these tests fell gradually with the progress of the disease. Contrary to this finding of the non-treponemal group of tests, the sensitive tests of the treponemal group (RPCF and FTA) manifested their reactivities till the later stages of the disease. Thus, the FTA test showed positive results with $gamma_1$ and $gamma_2$ globulins up to 48 weeks after infection, although reactivity with the RPCF test disappeared earlier. The reactivities of alpha and beta globulins with these tests also lasted longer in comparison with the treated group of rabbits but alpha globulin became negative earlier than the beta globulin in the later stage of the disease.

The TPI test showed positive results with the $gamma_2$ globulin of fraction 3 throughout the period of observation.

The difference in serologic reactivity of the serum globulins from the treated and untreated groups of rabbit depends on the antibody titre of serum during the progress of the disease. In the treated rabbits, the reactivities disappear earlier from all the globulin (alpha, beta, $gamma_2$ and $gamma_1$) fractions in successive order with the fall of serologic titre whereas in the untreated rabbits, the reactivities persist with the $gamma_1$ and $gamma_2$ globulins till the end of observation, although such reactivity manifested with alpha globulin disappear earlier than that of beta globulin fraction.

In these late stages of the disease, the disappearance of reactivities in globulin fractions with the different serologic tests depends mainly on the relative sensitivity of each test as less sensitive tests like the WR, KT, VDRL become negative earlier than the RPCF and FTA tests which showed more sensitive results. The TPI test showed a positive result only with the $gamma_2$ globulin in both the group of rabbits at this stage of the disease.

(2) Late stage of Human Infection (Table XXIII)

Six sera from the untreated tertiary and latent stages of syphilis were included in this investigation to observe the serologic reactivities of their globulin fractions at this late stage of the disease. All the globulin fractions from these 6

sera manifested reactivity with one or more of the serologic tests (WR, KT, VDRL, RPCF, FTA) depending upon their relative sensitivity although the TPI test showed a positive result only with gamma$_2$ globulin in each serum.

When the serologic reactivities of the 4 globulin fractions (alpha, beta, gamma$_1$, gamma$_2$) are compared in the untreated late stage of experimental rabbit syphilis and human infection, it is noticed that all these globulin fractions show positive results with one or more of the serologic tests (WR, KT, VDRL, RPCF, FTA) in human serum whereas the disappearance of such reactivity from alpha and beta globulins was noticed at this late stage of experimental rabbit syphilis, leaving the activity with gamma$_1$ and gamma$_2$ globulins for a prolonged period.

IV. CONGENITAL SYPHILIS (Table XXIII)

The serum from the case of congenital syphilis showed positive results only in gamma$_2$ globulin fraction with all the serologic tests used in this study. Even the most sensitive FTA test was unable to detect any antibody in other globulin fractions.

The results of these serologic reactivities with the globulin fractions of serum at the different stages of syphilis

in experimental disease of rabbits and in human infection are summarised as follows:

(a) Experimental rabbit syphilis: With the onset of infection, the $gamma_1$, $gamma_2$ and beta globulins show positive results with all the serologic tests (WR, KT, VDRL, RPCF, FTA) except the TPI which shows reactivity with $gamma_2$ globulin only at a later period when the test becomes positive at a considerable titre with the original serum. In the advanced stage of the disease, the reactivities of the more sensitive serologic tests (WR, VDRL, RPCF, FTA) spread to alpha globulin but the TPI test remains positive with the same $gamma_2$ globulin at this stage. In the late stage of infection, the treated group of rabbits show earlier disappearance of reactivity from alpha, beta, $gamma_2$, $gamma_1$ globulins in successive order. The less sensitive tests like the WR, KT, VDRL show negative results with these fractions earlier than the sensitive tests like RPCF and FTA as the antibody titre of the serum is lower with the former group of tests than with the latter. In comparison with the treated group of rabbits, the serum fractions of the untreated group show reactivity with sensitive tests (RPCF and FTA) in $gamma_1$ and $gamma_2$ glboulin till 48 weeks after infection; reactivity disappears earlier from alpha and

then from beta globulin fractions. The less sensitive tests (WR, KT, VDRL) show negative results in alpha, beta, $gamma_2$ and $gamma_1$ globulins in a successive way much earlier than the sensitive test (RPCF, FTA) in this group of rabbits, but in comparison with the treated group of rabbits, loss of reactivity occurs at a later period. The TPI test at this late stage of the disease remains positive in the same $gamma_2$ globulin till the end of 48 weeks of infection in the untreated group but the reactivity disappears from this fraction to a considerable level.

(b) Human Infection: The early stage of syphilis shows reactivity in $gamma_1$, $gamma_2$ and beta globulin fractions of sera, which is manifested differentially by the different serologic tests (WR, KT, VDRL, RPCF, FTA) depending upon their relative sensitivity but the TPI test shows negative results with all the globulin fractions at this stage of the disease as this test gives negative or doubtful results with these sera.

This finding of serologic reactivity in $gamma_1$, $gamma_2$, and beta globulins in the early stage of syphilis, does not correspond with the reports of Laurell (1955) and Ottolenghi (1958) who found the reagin only in gamma globulin at this stage of the disease. The difference may be due to observation of primary cases at a later period of infection in this

study.

In the secondary stage of the disease, the reactivity of WR, KT, VDRL, RPCF and FTA tests are also confined to the same $gamma_1$, $gamma_2$ and beta globulins but the less sensitive tests (WR, KT, VDRL) show stronger reactivity in these fractions in comparison with their results in the early stage of infection corresponding with their higher reactivity with the sera from secondary cases. In this stage, the TPI test becomes positive only with $gamma_2$ globulin at a time when this test shows a high titre with the sera.

In the secondary syphilis, Laurell (1955) and Ottolenghi (1958) mentioned the similar distributions of reagin activity in beta and gamma globulin fractions of sera. Laurell and Hederstedt (1958)also detected the TPI antibody in gamma globulin although the authors did not mention the particular gamma globulin responsible for the reactions.

In the untreated late stage of syphilis, the reactivity of serum fractions spreads from the previous position ($gamma_1$, $gamma_2$, beta globulins) to alpha globulin fraction but the presence of antibody in this fraction is detected mainly by sensitive tests like RPCF and FTA although the less sensitive tests like the WR, KT, VDRL could detect such antibody when the sera showed a high antibody titre with these tests. The

TPI shows a similar positive result with gamma$_2$ globulin as is found in the earlier stage of the disease.

This migration of antibody reactivity in the late stage of syphilis to alpha globulin was also reported by Ottolenghi (1958) but Laurell (955) found the activity of reagin only in beta$_2$ globulin at this stage of the disease and later Laurell and Malmquist (1961) detected the same reagin in both beta$_2$ M (gamma$_1$) and gamma$_2$ globulins although a component of the alpha globulins was mixed with the former fraction. Disappearance of reactivity from the latter fraction was also noted by these authors in some cases.

In congenital syphilis, the serologic reactivities with all the tests (WR, KT, VDRL, RPCF, FTA, TPI) remain confined to the gamma$_2$ globulin fraction of the serum. This restriction of serologic reactivity to gamma$_2$ globulin may be explained by the low antibody titre of the whole serum which prevented the appearance of such reactivity in other fractions; even the most sensitive FTA test was unable to detect any antibody in other globulin fractions. No conclusion can be drawn regarding this seroreactivity pattern of congenital syphilis as the observation was based on the finding of the single serum sample.

When the serologic tests are considered they behave similarly with the different serum protein fractions from both groups of rabbit syphilis and human infections. The TPI test reacts with the same gamma$_2$ globulin and other tests (WR, KT, VDRL, RPCF, FTA) behave similarly in showing positive results with all the globulin fractions, although the manifestation of such reactions depends on the sensitivity of individual serologic tests. But when the reactivity patterns of the different globulin fractions are considered throughout the disease process separately in both these groups, some fundamental differences are noticed which reflect the difference in the nature of the disease process in rabbits and human beings. These main differences are the quick spreading of serologic reactivities to the alpha globulin fraction in the advanced stage of the disease in untreated rabbit syphilis from its initial reactivity in gamma$_1$, gamma$_2$ and beta globulins followed by the quick disappearance of such reactivity from the alpha and beta globulin fractions. In contrast, the migration of the serologic reactivity to alpha globulin is manifested only in the late stage of human infection and reactivities in the alpha and beta globulins are a common occurrence in all the untreated late stages of syphilis in the human being.

This quick spreading of serologic reactivities to all the

globulin fractions and subsequent rapid disappearance of such reactivity from alpha and beta globulins in untreated rabbit syphilis may be related to the fact that in experimental infection of the rabbit, the disease process is local in nature, runs a rapid course and ends within a very short time. The localised nature of the disease in the testes was confirmed by histological examinations of autopsy specimens from different organs including the testes from a rabbit after one month of infection. The testis showed a granulomatous reaction (Fig. 13a, b) but other organs were found to be normal. A similar opinion was also expressed by Hollander and Turner (1954). The rapid course of this local infection was confirmed by the same histological examination of the testis from another rabbit one year after infection where lesions were found to be completely healed (Fig. 15a, b). This rapid course of the testicular lesion was also discribed by Browne and Pearce (1920).

In contrast to rabbit syphilis, the spreading of serologic reactivity to alpha globulin fraction in the late stage of human infection and maintenance of such reactivity in alpha and beta globulins could be explained as follows:

Due to a long continued infection in the human being, the antibody producing system is stimulated to its maximum and cells producing the alpha globulin also take part in such antibody

production and maintain such activity along with the beta and gamma globulins producing cells. This observation also suggests the possibility of multicellular origin of antibody production.

Guided by these reactive patterns of the different globulin fractions at the different stages in experimental rabbit syphilis and human infection, the reactivity pattern of globulin fractions from biologically false positive sera have been studied with the different serologic tests and using the reactivity of globulin fractions from normal sera as control.

Serologic reactivity of different globulin fractions in:

(1) Normal human sera (Table XXII)

Samples of 4 sera from normal healthy adults were fractionated for studying their serologic activity with the 6 serologic tests (WR, KT, VDRL, RPCF, FTA, TPI). The four globulin fractions did not show any reactivity with any of these serologic tests. This result is a contradiction of the earlier observations of Laurell and Malmquist (1961) who found a positive Wassermann reaction in gamma$_1$ (macroglobulin) globulin fraction using cardiolipin and crude heart extract antigens. These differences in observation regarding the reactivity of WR (Price's) may be due to the less sensitive

nature of this test.

(2) Biologically false positive sera (Table XXIV)

Fresh samples of serum were collected from 13 cases out of the group of 57 biologically false positive reactors (determined previously for the study of specificity of serologic tests in part 1) to study the serologic reactivity of their globulin fractions.

As the titre of reagin in this group of sera is very low, the globulin fractions were concentrated to double strength before carrying out the different tests but even after this increase of concentration, the serum fractions showed negative results with tests which gave a positive or weak positive result at 1:2 dilution with the whole serum. This behaviour prevents the appearance of uniform results in the pattern of reactivity of the globulin fractions in this series. However, the results of this study have demonstrated serologic reactivity in the $gamma_2$ globulin fractions of 9 sera out of 13 samples. In one serum sample, the reactivity was present in both $gamma_1$ and $gamma_2$ globulins whereas in another serum only the $gamma_1$ globulin showed a positive result. Two sera from this group did not show any reactivity with any of these globulin fractions as the titre of the reagin was low in the original serum.

The appearance of inconsistent serologic activity in the gamma globulin fractions prevents any definite conclusion about the nature and cause of the biologically false positive reactions with the non-specific tests like WR, KT, and VDRL in comparison with the reactivity of such fractions in the syphilitic sera. Similar inconsistent results were also noticed by Laurell and Malmquist (1961) who observed the serologic reactivity in gamma$_1$ (macroglobulin) globulin with the Wassermann reaction in all the biologically false positive sera studied by them whereas only a few sera showed such reactivity in gamma$_2$ globulin. The difference in findings cannot be explained properly unless the sources of such biologically false positive sera are known, as the authors failed to mention the names of the diseases. However, if these sera were collected from cases of infectious diseases like tuberculosis and leprosy, where tissue destruction is a prominent feature, there is every likelihood of getting a regular appearance of reagin in the macroglobulin fraction and migration of the reagin to gamma$_2$ globulin may be noticed in some cases. This phenomenon may be due to a persistent stimulus to antibody-producing cells by the tissue lipoid unmasked by the infective process which affected the macroglobulin-producing

cells earlier followed by the gamma$_2$ producing cells of the reticulo endothelial system. But if the authors tested those sera from biologically false positive reactors of connective tissue disorder, one cannot explain the regular appearance of reagin in the macroglobulin fraction. Because in systemic lupus erythematosus where the bulk of antibody is present in gamma$_2$ globulin, one should expect the regular appearance of reagin in this fraction.

However, it is apparent from this study that the serologic reactivity of the globulin fractions in biologically false positive sera is restricted to the gamma globulin fractions (gamma$_1$ and gamma$_2$) and this gamma globulin behaves similarly in electrophoretic mobility with the same fraction from syphilitic and normal sera.

GENERAL DISCUSSION

Chapter X

GENERAL DISCUSSION

The result of the evaluation study of the different serologic tests reported in the Serology, Evaluation and Research Assembly (SERA) under the auspices of the United States Public Health Service in 1957, showed the promising future of the fluorescent treponemal antibody (FTA) and Reiter's protein complement fixation (RPCF) tests, as these tests were found to give sensitive and specific results and compared favourably with the TPI test. Tests using cardiolipin antigen were also found to have some place next to the treponemal tests. Although the present evaluation study was carried out mainly in experimental rabbit syphilis, the results show similar findings to those reported in SERA which were carried out on human sera. This suggests that so far as the antibody pattern of the serologic test is concerned, the rabbit and human behave similarly as was mentioned before by Turner and Hollander (1957). Apart from this general finding suggesting the relative importance of the FTA-200, TPI, RPCF and VDRL tests in this evaluation study, each test also showed its characteristic behaviour in relation to its sensitivity and specificity as follows:

FTA-200 test: This test shows the most sensitive results in the early and florid stages of syphilis. In the late untreated

stage, the reaction is as sensitive as the TPI test but in the treated late stage of syphilis, the FTA test becomes negative some time after the cessation of treatment and could be used as a test for cure. Like the TPI test, it could eliminate a biologically false positive reaction.

A similar result of specificity and sensitivity of the FTA-200 test was also noticed by Wilkinson (1961) when he evaluated the result of the FTA-200 test in comparison with the WR, RPCF and TPI tests in the sera from normal, treated and untreated treponemal diseases and biologically false positive reactors.

TPI test: The TPI test is sensitive enough to detect syphilis in all stages beyond its early primary stage. Thus the test could be used as a diagnostic test except in early primary syphilis. As this test remains positive indefinitely in the late stage of syphilis after treatment, it is not suitable for use as a test for cure. The TPI test eliminates biologically false positive reactions as it is universally accepted as a specific test for syphilis. Similar opinion was also expressed in the past by various workers (Magnuson and Thompson, 1949; Mohr et al. 1959; Nelson et al., 1951; Chacko, 1953; Edmudson et al. 1954; Wilkinson, 1954; Sequiera, 1955; Neilsen and Rein, 1956).

RPCF test: This test shows as sensitive results as the

VDRL test in early cases of syphilis; it is as sensitive as the VDRL and TPI tests in the florid stage of the disease and remains positive in late untreated syphilis for a long time like the TPI, FTA and VDRL tests. In treated late syphilis, it becomes negative earlier in comparison with the VDRL and FTA tests.

Although the RPCF test was claimed to be more sensitive and as specific as the TPI test by various workers (De Bruijn, 1956; Cannefax and Garson, 1957; Rein et al., 1958; Miller et al., 1958; Foster et al., 1958, 1959; Sequeira, 1959; Wilkinson and Johnston, 1959), it also shows some non-specific results (Berner et al., 1960). But the specificity is found to be better than the VDRL test.

VDRL test: This test shows similar sensitivity to the RPCF test in the early cases of syphilis, and manifests sensitive results similar to the TPI and RPCF tests in the florid stage of the disease. In late untreated cases, the test remains positive for a long time after infection like the TPI and FTA tests, but at a low titre; whereas in treated late cases of syphilis it becomes negative earlier than the FTA test. The non-specific results revealed by this test are found to surpass the results of the RPCF test.

Edmudson et al. (1954) also found a similar sensitivity with the VDRL test when they compared this test with the TPI at different stages of syphilis. The National Serological Evaluation Servey (1949) sponsored by the United States Public Health Service proved the occurrence of a non-specific reactions with the VDRL test similar to other non-treponemal tests.

Although the FTA-200 test is found to be the most sensitive serologic reaction amongst all the tests used for this study, it does not show positive results with all the sera from early cases of primary syphilis. So an effort has been made to increase the sensitivity of the FTA-200 test by lowering the testing dilution of the serum from 1:200 to 1:25 so as to make the test sensitive enough for the detection and confirmation of early cases of primary syphilis. However, the expected sensitivity could be obtained by using the FTA-25 test only with an appreciable reduction of its specificity. Even with this loss of specificity, the FTA-25 test could probably be used for the diagnosis of suspected cases of early syphilis when the dark-ground examination is not available to clinicians and the risk of getting a biologically false positive reaction in the serum could be excluded by the most non-specific test like the KT. For example, a negative Kahn reaction with a

positive FTA-25 test may help in diagnosing such suspected early cases of syphilis.

The peculiar finding of the most specific FTA-200 test showing a non-specific reaction at a lower dilution with normal human and biologically false positive sera (Table XV, XX) could be explained as follows:

In syphilis, two types of antibody (antilipoidal or reagin and antitreponemal) appear in serum against lipoid and protein components of treponemes. The origin of reagin may also be considered as an antibody response against tissue lipoid due to destruction of tissue by treponemal infection. But in biologically false positive sera, reagin which is presumably responsible for the non-specific reaction, appears in a low titre. Most of the individuals showing such reactivity in their sera, are usually found to have systemic diseases after thorough clinical and laboratory investigations. The acute biologically false positive reactions are attributed to a variety of systemic infections, bacterial, viral, rickettsial or protozoal. They appear during or subsequent to such diseases and regress spontaneously to normality within a relatively short period of time, not exceeding six months. Chronic biologically false positive reactions usually persist for a

period of years and perhaps a life time. They are not due to such precipitating causes (except a few like lepromatous leprosy and chronic pulmonary tuberculosis) as are seen in the acute biologically false positive reactions but reflect the presence of or predisposition to chronic systemic diseases like the connective tissue diseases (e.g. systemic lupus erythematosus and rheumatoid arthritis) which might otherwise go unrecognised.

The FTA test detects both the reagin and anti-treponemal antibody in syphilitic serum as the test remains positive with such serum even after removal of reagin by repeated absorption with the cardiolipin antigen. Detection of these two types of antibody by the FTA test was also reported by Wilkinson (1961). The increased sensitivity of the FTA test with the syphilitic serum is probably due to detection of both the so-called complete and incomplete reagin and antitreponemal antibody. Coombs serum used in fluorescein conjugate helps to detect the incomplete antibody. Moreover, this conjugate also detects the complete antibody usually detected by other serologic procedures. Thus the sensitivity of the FTA test becomes so high in comparison with the other serologic tests in all the stages of syphilis that by using a testing dilution of serum at 1:200, the non-specific reaction in biologically false positive

and normal human sera could be avoided, as this reaction is presumably due to the presence of reagin and appears in low titre.

From the study of experimental rabbit syphilis, it has also been noticed that the antitreponemal antibody detected by the FTA test is not similar to the TPI antibody (immobilisin) as in the treated cases of late syphilis, the TPI test remains positive when the FTA test shows a negative result.

From the results of this evaluation study, it could be concluded that the FTA-200 test could be used routinely as a specific diagnostic test for syphilis in place of the TPI test. This test has the advantages that (a) it is more sensitive than the non-treponemal group of tests in early cases of syphilis; (b) it is as sensitive as the TPI test in the late stage of syphilis; (c) it becomes negative earlier than the TPI test in the treated late cases so that it could be used as a test for cure; and (d) like the TPI test, it eliminates biologically false positive reactions.

Technically the FTA-200 test has the great advantages over the TPI test in that the dead treponemal antigen could be made available from a Central Reference Laboratory along with other reagents; the antigen could be stored in the

refrigerator for a period of 6 months or more; the slides used for the test could be preserved in the cold for future reference purposes and there is no risk of toxic or anticomplementary effects from the serum used for the test. Also, slightly contaminated serum could be used for testing purposes.

The elaborate study of the serologic reactivity in the serum protein fractions from normal, syphilitic and biologically false positive sera revealed the results of different patterns of reactivity in these groups. But these patterns of reactivity could not help in differentiating these groups of sera from each other when the non-specific serologic tests (WR, KT, VDRL) are used for the seroreactivity of their globulin fractions. This is due to an inconsistent finding of the serologic reactivity with the $gamma_1$ and $gamma_2$ fractions from the biologically false positive sera. When both these fractions show negative results, the reactive pattern of biologically false positive serum simulates that of normal serum, but a positive result with $gamma_1$ or $gamma_2$ or both the fractions from such serum, resembles the pattern of syphilitic serum (treated or untreated late stage). The appearance of a similar type of reactivity in the globulin fractions from the syphilitic and biologically false positive sera also prevented earlier workers (Coburn and Moore, 1943; Laurell, 1955; Laurell

and Malmquist, 1961) from identifying these sera from such serologic patterns.

However, the character of the reagin, which is presumably responsible for the reactivity in biologically false positive sera, could be ascertained to a certain extent from this study. This reagin occurs in gamma globulin fractions (gamma$_1$ or gamma$_2$ or both the globulins) which show similar electrophoretic mobility to that of syphilitic and normal serum. Thus the reagin should be considered as a part and parcel of the gamma globulin molecule as Laurell and Malmquist (1961) mentioned clearly in the past.

A qualitative alteration of the gamma globulin molecules cannot be responsible for the reagin activity in biologically false positive sera as the gamma globulin fractions of these sera behave electrophoretically similarly to those of normal sera. This view is also supported by the fact that although the qualitative and quantitative character of globulin is changed in multiple myelomatosis, no such biologically false positive reaction is manifested in these sera.

But Moore and Lutz (1955) noticed a frequently associated earlier disorder of serum gamma globulin (dysgammaglobulinaemia) in cases of chronic biologically false positive reactors,

who were sometimes found later to develop diseases like systemic lupus erythematosus, rheumatoid arthritis or diseases with similar clinical manifestations. The authors used protein flocculation tests such as those for the detection of dysgammaglobulinaemia, so their suggestion about the mechanism of the false positive reaction could not be accepted as due to a qualitative alteration of gamma globulin molecules because a quantitative increase in this globulin fraction is usually found to be associated with a positive liver function flocculation test. But their observation on the frequent occurrence of the chronic biologically false positive reactions in individuals who later develop diseases like systemic lupus erythematosus and rheumatoid arthritis supports the hypothesis of a genetic factor in the development of these reactions, as does the finding of similar antibodies in the sera of the nearest relatives of patients with "connective tissue diseases".

Moore and Mohr (1952) noticed an occasional elevation of serum globulin with the shift of A/G ratio from cases of biologically false positive reactors after the exclusion of renal and hepatic disorder and explained the manifestation of the false positive reaction as being due to a quantitative increase of globulin. But the serologic reactivity of the

gamma globulin fractions could not be accepted as due to a quantitative increase of individual globulin content of serum ($gamma_1$ or $gamma_2$) like the flocculation tests of liver function where the A/G ratio determines such reactions. Although cases of rheumatoid arthritis and systemic lupus erythematosus are always associated with an increase of macroglobulin and $gamma_2$ globulin respectively, all such sera do not show false positive reactions. Similarly the sera from cases of chronic parenchymatous liver diseases or chronic bacterial infections like empyema or lung abcess are usually associated with hyperglobulinaemia but biologically false positive reactions are a rare occurrence in such cases.

So the activity of reagin in gamma globulin fraction could be postulated as due to the alteration in structural configuration of the gamma globulin molecule as found in antibody globulin or, in other words, reagin should be considered as an antibody. This antibody production may be caused by a stimulation of the reticulo endothelial system by the unmasked tissue lipoids in infectious diseases like tuberculosis and leprosy where tissue destruction is a prominent feature (Sequeira, 1959). But in connective tissue disorders (e.g. systemic lupus erythematosus and rheumatoid arthritis),

the establishment of "forbidden clones" associated with genetic mutation of cells in the reticulo endothelial system may produce an altered structural configuration of gamma globulin molecules, thus producing varieties of antibody including the reagin (Raffel, 1961).

SUMMARY

SUMMARY

The sensitivity and specificity of various types of serologic tests developed so far for the diagnosis of syphilis have been evaluated in this investigation. At the same time, the reactivity of these tests with the different globulin fractions from syphilitic and biologically false positive sera have been compared (using the same reaction with the globulin fractions from normal sera as a control) to find out the nature of false positive reactions and if possible to simplify its detection by easier non-treponemal serologic tests.

Study of sensitivity and specificity of serologic tests

The sensitivities of the newer serologic test (Reiter's protein complement fixation test - RPCF , Fluorescent treponemal antibody test - FTA and Reiter's fluorescent treponemal antibody test - RFTA) have been evaluated in different stages of experimental syphilis in rabbits (treated and untreated) and in the early stage of human infection in comparison with the Treponema pallidum immobilisation test (TPI) and non-treponemal tests (Wassermann reaction-WR - Price's, Kahn standard test - KT, Cardiolipin slide flocculation test - VDRL) as the sensitivities of these latter tests in different stages of the disease are well documented.

The specificity of all these tests have been studied in 57 biologically false positive sera from patients with a variety of clinical diseases.

The results of this evaluation of the sensitivity and specificity of these tests show their importance in the following order:

1. FTA test: FTA-200 test is found to be the most sensitive in the early stage of human syphilis. In experimental rabbit syphilis, the FTA test shows the most sensitive result in early and advanced stages of the disease. In the late untreated cases, the test remains positive indefinitely like the TPI test whereas in the treated case it becomes negative a long time after treatment and thus could be used as a test for cure. Like the TPI, the FTA-200 test also eliminates biologically false positive reactions.

Technically the test has advantages over the TPI as the dead treponemal antigen could be made available from a Central Reference Laboratory and the antigen can be stored in the refrigerator for 6 months or more without losing its potency. The FTA test can be used with anticomplementary and contaminated sera.

An attempt to increase the sensitivity of the FTA-200 test by lowering the testing dilution of sera to 1:25 has been

successful as the FTA-25 test shows positive results with all the sera from early cases of human syphilis in comparison with the FTA-200 test. But the specificity of the FTA-25 test is found to be poor as the test shows non-specific results with most of the sera from biologically false positive reactors whereas the FTA-200 test shows absolute specificity. However, the possible application of the FTA-25 test has been suggested for diagnosis of suspected early cases of syphilis when the facility for darkground examination is not available and where the possibility of biologically false positive reaction could be eliminated by a negative Kahn test.

2. TPI test: This test is less sensitive than the FTA test in the early stage of human and experimental rabbit syphilis. In the advanced and late untreated case of experimental rabbit syphilis it reaches a high titre whereas in the treated late stage of the disease in rabbits, this test has little value as it remains positive indefinitely and cannot be used as a test for cure. As the TPI test is considered universally to be a specific test for syphilis, it is used as a standard specific test for this evaluation study.

Technical difficulties such as maintenance of treponemes in rabbit testes, use of costly reagents for the survival medium and maintenance of strict asepsis during testing

procedure, restrict the use of this test to a well equipped laboratory.

3. RPCF test: This test shows a degree of sensitivity similar to the non-treponemal group of tests in the early stage of both human and experimental rabbit syphilis. In the advanced stage of rabbit syphilis, it shows a reasonable sensitivity in comparison with other tests. In late untreated syphilis of rabbits, the test remains positive for a prolonged time whilst in the treated late stage it becomes negative earlier. Although it shows non-specific reactions, the incidence is found to be lower than that of the non-treponemal group of tests.

4. VDRL test: Although this test shows non-specific results in biologically false positive sera in a greater percentage of cases than the WR (Price's) it gives more sensitive results than the latter test in early experimental rabbit syphilis and a sensitivity similar to WR, is found in early cases of human syphilis. This test maintains a high titre in the advanced stage of rabbit syphilis and remains positive in late untreated cases of this disease. In the treated late stage of syphilis in rabbits, the test becomes negative earlier than the FTA test.

5. WR (Price's): In spite of a more sensitive result than

the TPI in the early stage of both human and experimental rabbit syphilis and a similar sensitive result to all other tests in the advanced stage of the disease in rabbits, the WR becomes negative earlier than all other tests both in the treated and untreated late stage of syphilis in rabbits. But this test shows less tendency to give non-specific results than the VDRLwith the biologically false positive sera.

6. KT: This test is more sensitive in the early stage of experimental rabbit syphilis than is the WR (Price's); but in early human infection it shows similar sensitive results. In the advanced stage of syphilis in rabbits, KT shows the least sensitive result and in the late stage of this disease, the test becomes negative earlier both in treated and untreated cases in comparison with the VDRL test. KT shows the maximum percentage of non-specific reactions amongst all the tests included in this study.

7. RFTA test: In experimental rabbit syphilis, the test shows sensitivity similar to KT in all stages of the disease. But this RFTA test at 1:200 dilution is found to be the least sensitive test in this study as it shows negative results with all the sera from early cases of human syphilis where all other tests show some positive results. So its apparent specificity with biologically false positive sera has not been

considered as the test shows the poorest sensitivity.

Guided by this result of sensitivity and specificity of the FTA-200 test and from the points of its technical superiority over the TPI test the FTA-200 test could be recommended as the standard specific serodiagnostic test for syphilis in place of the TPI test.

Study of seroreactivity in serum protein fractions

Sera from cases of human and rabbit syphilis in different stages, sera giving biologically false positive reactions and normal sera have been fractionated into 4 globulin components (gamma$_1$, gamma$_2$, beta, alpha) by column chromatography on DEAE-Sephadex, to find out the serologic reactivity of these globulin fractions. The reactive pattern of these globulin fractions with the WR, KT, VDRL, RPCF, FTA and TPI tests are found to be as follows:

Experimental rabbit syphilis

Along with the rise of antibody titre in the sera at the onset of the disease, the serologic reactivity appears in gamma$_1$, gamma$_2$ and beta globulin fractions with all the tests except the TPI and spreads to the alpha globulin fraction in the advanced stage of the disease, whilst the TPI test reacts only with gamma$_2$ globulin. In the treated late stage of the disease, the reactivity disappears quickly from all the frac-

tions in successive order from alpha, beta, $gamma_2$ and $gamma_1$ globulins; the non-treponemal tests showed negative results earlier in these fractions in comparison with the treponemal group of tests. The TPI test maintains its reactivity in $gamma_2$ and disappears from this fraction when the titre becomes low in the serum. In the untreated stage of syphilis, all the treponemal tests remains positive in gammal and $gamma_2$ globulins till 48 weeks of observation in comparison with the non-treponemal group but the TPI reactivity is found to be restricted in the same fraction as the treated group. Disappearance of reactivity from the alpha and beta globulins in this untreated late stage of syphilis has been noticed earlier with the non-treponemal group than the newer treponemal group of tests (RPCF and FTA).

Natural human infections:

In untreated human syphilis at different stages of infection, reactivity of the different globulin fractions with all the serologic tests has behaved rather similarly to that in the untreated group of diseased rabbits. The migration of reactivity to the alpha fraction with all the serologic tests (except the TPI) has been noticed only in the late stage of the disease and serologic reactivity persists in all the globulin fractions at this stage. In one case of congenital human syphilis, the

reactivity of all the serologic tests was found only in the gamma$_2$ globulin fraction.

Normal human and biologically false positive sera:

When the reactivi ty of the serologic tests with the globulin fractions from syphilitic sera are compared with the same globulin fractions from normal human and biologically false positive sera, no reactivit y is found with the normal group of sera whereas in the latter group, the reactivity of the non-specific WR, KT, and VDRL tests is found to be restricted to the gamma globulin. This gamma globulin shows similar electrophoretic mobility to those of normal and human syphilitic sera. But the distribution of reactivity to gamma$_1$ and gamma$_2$ globulins varied in different biologically false positive sera used for this study; serologic reactivity with the gamma$_2$ fraction was found in the majority of the sera and a few sera showed such reactions either with gamma$_1$ alone or with both gamma$_1$ and gamma$_2$ fractions. An absence of reactivity from both these fractions was also noticed in a few sera when the serologic tests showed a poor titre.

From these results it is concluded that although the serologic reactivity of globulin fractions from normal, syphilitic and biologically false positive sera are known, it is not possible to differentiate these sera from the results of such seroreactivity with the non-specific tests like the WR, KT and

VDRL as similar types of reactivity are occasionally found in all these groups of sera.

However, the character of the reagin, which is presumably responsible for the reactivity in the biologically false positive sera could be ascertained to a certain extent from this study. The presence of the reagin in the gamma globulin molecules, with a similar electro-phoretic mobility to those of human and syphilitic sera, suggests it to be a part and parcel of this gamma globulin. The manifestation of its serologic reactivity has been considered as probably due to an alteration in the structural configuration of the gamma globulin as found in antibody globulin or in other words reagin should be considered as an antibody. The antibody production may be caused by a stimulation of the antibody-producing cells by the unmasked lipoid as found in tuberculosis or leprosy or it appears in the serum as one of the manifestations of abnormal antibody production as found in connective tissue disorders like systemic lupus erythematosus and rheumatoid arthritis.

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