(1) THE PREPARATION AND VALUE OF A STANDARDISED MODIFICATION OF THE LAUGHLEN TEST FOR SYPHILIS

(2) INVESTIGATIONS ON SYPHILIS ANTIGEN:

(a) The Value of Soya Bean Extracts

(b) The Significance of the Diaminomonophosphatide Sphingomyelin

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Contents

Introduction

The work recorded herein developed from an investigation into the use of a rapid serological test for syphilis. In recent years many such tests have been investigated by laboratory workers owing to the great increase in the number of specimens sent for serological examination - an increase which has placed an immense strain on most of the laboratories dealing with serological work and particularly on public health laboratories where the volume of specimens marked "for W.R." may at times reach enormous proportions. The performance of a Wassermann Reaction on every specimen frequently becomes a laborious and almost impossible task since the test is comparatively lengthy and delicate and requires experienced supervision at every stage. The result has been a search for tests more easily and more rapidly performed than the Wassermann, capable of being carried out with simple apparatus and having a degree of sensitivity sufficient to identify all specimens giving a positive result with the complement-fixation test.

With the improvement in the technique of the many quickly-performed precipitation tests for syphilis described during the past twenty-five years, there came the hope that the serological diagnosis of this disease would become an extremely simple, swift and reliable process, and when it became increasingly obvious that precipitation tests were valuable diagnostic aids, many serologists even predicted that the Wassermann Reaction would fall into disuse. Far from this, however, the

Wassermann has become still more firmly established as the standard method of confirmation in an investigation for syphilis and even when a diagnosis of the disease has been reached by other methods few workers, either in the clinic or laboratory, would be content to omit examination of the blood by the Wassermann Reaction. This faithful adherence to the results of the complement-fixation test is not to discredit the precipitation tests but is most desirable in that it introduces a certain measure of standardisation in the methods of diagnosis.

Accordingly, it may be argued that a convenient method of diagnosing syphilis in a busy laboratory would be to examine the specimens under investigation first of all by one of the comparatively rapid precipitation tests. The sera giving negative results with this preliminary testing could be reported as such to the clinician concerned without further examination and those giving doubtful or positive reactions retained for confirmation by the Wassermann. The advantages of such a system are obvious. Much time is saved by the sorting-out or "screening" of large numbers of sera by a rapid test and also fewer specimens require to be examined by the time-consuming complement-fixation reaction. Alternatively, the initial rapid test could be carried out in small laboratories where the Wassermann might be impracticable and specimens for further testing forwarded to a larger centre.

The basis of this screening system depends on one all-important fact - the reliability of the precipitation test employed. It is essential that complete confidence can be placed in the negative results obtained with this test since these are reported without confirmation by other methods. In this instance sensitivity is vastly more important than selectivity, remembering that the purpose of the preliminary screening is to detect those sera which are definitely non-syphilitic or in other words, the sensitivity of the precipitation test must be much greater than that of the reaction, usually the Wassermann, which is used later in confirmation of doubtful and positive results.

Several precipitation reactions are now used as preliminary screen tests. The best known in this country is probably the Presumptive Kahn Test and in America quite a number have come into use in the last few years, e.g., the Kline Test, the Hinton, the Mazzini and others, including, of course, the Presumptive Kahn. In 1935, Laughlen, in Canada, described a rapid precipitation test which may be placed in the same category. Fundamentally, this reaction can be considered as a modified Kahn Test, since Kahn antigen was the basic constituent of the Laughlen antigen, but it was performed on slides in place of tubes and a novel innovation was the incorporation of a dye which rendered the precipitated particles more easily seen. Many reports on the Laughlen Test have been made by workers both in America and Britain and it is evident that the reaction has been found by the majority to be a reliable aid in the diagnosis of syphilis, its most notable features being simplicity of performance and the rapidity with which large number of sera can be tested. Several modifications of the test have been described without altering its essential structure.

The original purpose of the present investigation was to determine the suitability of the Laughlen Test for the routine examination of ante-natal specimens. As a result of the enquiry a new modification of the reaction was produced and subjected to extensive trials which indicated that this version of the test possessed high levels of sensitivity and accuracy under certain conditions. The speed of the test's performance made it an excellent method of conducting research into problems concerning syphilis antigens and this feature was made use of in the other work described in this thesis.

Some time before the completion of the investigation into the use of the Laughlen reaction as a screen test, the possibility of extracting antigenic substances from materials other than the customary heart muscle was considered; the particular substances contemplated were nervous tissue and certain plants, since the former is known to be a rich source of the lipoids forming the active portion of heart-muscle antigens and appreciable quantities of lipoids are present in vegetables. One drawback to this projected research was the length of time necessary if the Wassermann Reaction were to be used as the indicator of antigenic activity in extracts made from the substances mentioned above. The development of the modification of the Laughlen Test now suggested a convenient method of rapidly assessing the antigenic value of any such extracts by using them in place of the usual Kahn antigen basis of the modified Laughlen re-agent. By this method, examination of a phosphatide extracted from human brain yielded considerable information concerning the mode of action of the complex organic compounds constituting so-called syphilitic "antigen" and similarly it was established that antigens for precipitation tests for syphilis can be extracted from a vegetable which already has been put to innumerable uses - the soya bean.

The records of the various investigations are preceded by a brief review of the history of the syphilitic antigen since much of the work centres round this mysterious substance. This is followed by two sections, the first of which describes the steps leading to the present modification of the Laughlen Test; the second contains a description of how this version of the test was used as an instrument of research, i.e., in the enquiries into the antigenic activity of certain extracts made from nervous tissue and the soya bean.

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Historical Notes on the Development of Syphilis Antigen

Syphilis "antigen" is one of the most puzzling substances in the entire field of medical science. It is difficult to think of another instance where an antigen used in the diagnosis of a particular disease is so completely unrelated to the pathogenesis of that disease or indeed of any disease. Even the somewhat strange agglutination of the proteus bacillus by the serum of a typhus patient is not so mysterious as the reactions of the non-specific antigen for syphilis, yet this antigen provides the basis for tests which are among the most reliable in modern laboratory technique. Forty years have passed since it became known that the "antigens" used in tests for syphilis were not true antigens but there is still no clear understanding of their action in either the complement fixation or precipitation which they produce with syphilitic serum.

Until 1906 there existed no serological test for the diagnosis of syphilis. In that year Wassermann, Neisser and Brück published details of such a test based on the phenomena of haemolysis of red blood corpuscles by antisera (Bordet, 1898), and complement fixation (Bordet and Genou, 1901). Although there are good grounds for believing that the credit for first describing the use of complement fixation in a test for syphilis in the human subject by right belongs to Detre, whose work in this connection was also published in 1906, this method of testing for syphilitic infection is now universally known as the Wassermann Reaction. Complement, a natural constituent of serum, had been the subject of much scientific enquiry up to this time and two all-important facts had emerged to prepare the way for the elaboration of the Wassermann Reaction - (1) complement must be present before red blood corpuscles can be haemolysed by their specific anti-serum; and (2) this haemolytic property of complement, in addition to being destroyed by heat, is inhibited or "fixed" when the complement is subjected to the interaction of an anti-body with its specific antigen. It followed that, given a specific antigen, it was possible to test for the presence of the corresponding antibody by observing what happened to complement when it was placed in a mixture of the antigen and a substance under test. Red blood corpuscles, sensitised with their homologous antiserum, could be used as an indicator: haemolysis would indicate the presence of free complement which in turn would mean that the specific antibody had not been present; absence of haemolysis would show that the complement must have been "fixed" by the interaction of the antigen with its antibody, i.e., a positive result.

It seemed, therefore that all that was required for the production of a serological test for syphilis was a specific antigen, since all the other components necessary for a complement fixation test were already present, assuming, of course, that an antibody to syphilis was produced in the blood of an individual suffering from the disease. The identification of the treponema pallidum as the causal organism of syphilis (Schaudinn and Hoffmann, 1905) brought the hope that this specific antigen was now at hand since it was reasonable to suppose that this organism, in common with many other pathogenic microbes, could be used as an antigen for the disease it produced. In 1906, however, no reliable culture of the treponema was available and as an antigen for their test Wassermann and his co-workers resorted to what was probably considered the next best thing - an extract of the liver from a syphilitic foetus. This extract was made with water and when the new test began to produce results consistent with clinical findings, the obvious conclusion was that the water had removed from the liver the specific antigen of syphilis. Although this assumption has been proved to be fundamentally wrong it gave rise to a great volume of research on these tissue extracts and thus brought about an extremely rapid development of the test's potentialities. It is indeed fortunate that there were no pure cultures of the treponema pallidum in 1906, otherwise they would almost certainly have been used as the antigen for complement fixation tests and would have caused delay in the development of the Wassermann Reaction since it has been found (Schereschewsky, 1913, Kolmer, 1942) that as antigens they produce irregular and unreliable results.

The antigen-antibody theory of the Wassermann Reaction was destined to receive a serious setback only a few months after the test's inception. Early in 1907, a publication by Weygandt described an antigen made from normal spleen which had been used in a complement fixation test; this test had given positive results in definite cases of late syphilis. This finding was confirmed by many other workers and it soon became obvious that many normal organs could provide "antigens" for syphilis equally as effective as those derived from syphilitic tissue. That this should be so was puzzling in the extreme and the only answer to the problem was that the antigenic substance, supposed by Wassermann and his co-workers to have been present in their liver extract solely because of the syphilitic infection of that organ, must be a normal constituent of many organs of the animal body and as such could hardly be called a true antigen. The fact remained, nevertheless, that this unknown substance was capable of reacting <u>in vitro</u> with the antibody present in syphilitic blood in the same way as a true antigen, at least as far as concerned the phenomenon of complement fixation.

The first step towards discovering the real nature of this antigen-like substance was made in December, 1907, when Landsteiner, Müller and Potzl found that a satisfactory antigen for the Wassermann Reaction was provided by an alcoholic extract of guinea-pig heart and that the results obtained with its use were equivalent to those obtained with the original watery extract of syphilitic liver. This pointed to the fact that the "antigenic" principles were soluble in alcohol and provided the basis for widespread research directed towards discovering their chemical nature. During the next few years many reports on numerous antigen preparations were published until it appeared beyond all reasonable doubt that the antigenic action was associated with the normal lipoids which are present to a greater or less extent in all living tissue. Many individual lipoids were investigated in an effort to find the one responsible for the antigenic activity - lecithin (Porges and Meier, 1908), sodium taurocholate and glycocholate (Levaditi and Yamanouchi, 1907), cholesterin and vaselin (Fleischmann, 1908), oleic acid (Sachs and Altmann, 1908), and many other substances were subjected to intensive experiments to find what part, if any, they played in this peculiar non-specific reaction. It was found, however, that by far the best results were obtained when the antigen consisted of a tissue extract, i.e., a mixture of "crude" lipoids. These tissue lipoids were collectively referred to as "the lecithins" and later, more correctly, as "phospholipids" or phosphatides, further details and the chemistry of which will be discussed later in some detail (Page 101).

An important advance in the methods of using these "antigens" was made in 1910 when Browning, Cruickshank and

McKenzie announced the results of their work with extracts to which cholesterol had been added. Two antigens had been used in their experiments - alcoholic tissue extracts and alcoholic solutions of lecithin. The conclusions reached by these workers were (a) that the sensitivity of the so-called syphilitic antigens was markedly increased by the addition of cholesterol, and (b) that the best combination was lecithin and cholesterol. This sensitising effect of cholesterol was soon corroborated by the work of other investigators though the majority supported a later contention that even with this adjuvant the crude tissue extracts still produced the best results. (Sachs, 1911).

Five years had now elapsed since the introduction of the Wassermann Reaction and the mystery of the non-specific syphilitic antigen was still unsolved. The test was being improved and made more accurate almost daily but there was still no exact understanding of the process by which an alcoholic extract of a normal organ could act as an antigen to syphilis antibody in a complement fixation reaction. Certain important facts, however, had been established -

(a) Normal tissues produced antigens equally as satisfactory as those extracted from syphilitic organs;

(b) The antigens giving the best results were alcoholic tissue extracts; and

(c) The sensitivity of these antigens was greatly increased by the addition of cholesterol.

That these three principles were correct has been proved repeatedly in the period which has elapsed since they were formulated and at the present time most of the several modifications of the Wassermann Reaction employ as antigen an alcoholic tissue extract to which has been added cholesterol, the effect of which is to provide crystalline surfaces on which the antigen-antibody reaction takes place (Weil, 1941).

The subsequent history and development of syphilis antigen is closely connected with precipitation tests. Compared to the Wassermann Reaction, precipitation tests for syphilis are remarkable in that they developed slowly and did not become generally used until comparatively recent times. In some measure at least this was no doubt due to the amount of attention which was focused on complement-fixation. Nine years before the presentation of the Wassermann Reaction, Kraus (1897), working in Vienna, had published the first account of the phenomenon of precipitation which, so far as is known, had not been observed up to that time. Kraus's experiments were concerned with the microbes of cholera, typhoid and plague and he had found that when sterile filtrates from cultures of these organisms were added to their homologous antisera small floccules appeared in the mixtures. These "precipitates" he considered to be parts of the bacteria of the original cultures. Attempts were now made to apply this phenomenon to several diagnostic problems and as early as 1907, only a few months after the initial description of the Wassermann Reaction had been published, a report by Michaelis gave details of his attempts to produce precipitation with syphilitic sera. In his experiments, Michaelis had employed a watery extract of syphilitic liver as antigen and had observed precipitates when it was added directly to syphilitic serum. This work does not seem to have been fully appreciated at the time for several years were to pass before real progress was made in the practical application of a precipitation test to the diagnosis of syphilis. Three years later, 1910, Jacobsthal reported the use of an alcoholic tissue extract as an antigen in a precipitation test he had developed and in the following year Brück and Hidaka used a similar antigen for a test in which the mixtures of serum and antigen were centrifuged after incubation. Alcoholic extract of heart was used by Hecht who published an account of his work in 1914.

Up to this time precipitation tests for syphilis had not been accepted generally as being of value compared with the Wassermann Reaction. There were several reasons for this; precipitation tests required long incubation periods, the antigens used were often unsatisfactory and the results obtained showed a level of sensitivity considerably lower than that of the complement-fixation test. About 1918, however, an increased interest seems to have developed and in the course of a few years many of the problems which had obstructed the progress of the precipitation reactions were solved. The first advance came when Sachs and Georgi (1918) published an account of a test employing cholesterolised antigen and in the following year Meinicke described the treatment of dried tissue with ether before making the alcoholic extract which was used as antigen. By this method of "purification" he claimed to remove unstable substances from the dry tissue. (Similar attempts to purify the antigens for the Wassermann Reaction had been made by Noguchi in 1909 but crude extracts had been found to give better results.) Meinicke's work provided the basis for practically every precipitation test subsequently developed and the preliminary treatment of dried tissue with an organic solvent such as ether or acetone has become an integral part of the preparation of antigens for use in these reactions (vide "Chemistry of Syphilis Antigen", page 94).

In 1922 the Kahn Test was described and with it there were introduced two features which have become essential procedures of almost all precipitation tests for syphilis. These were (a) the use of minimal quantities of antigen: and (b) agitation of the serum-antigen mixtures instead of incubation which had been practised hitherto. The results obtained with the Kahn Test were so striking that it was obvious that precipitation tests had at last reached the point where they could be put to practical use. The serological diagnosis of syphilis had entered a new phase and it was evident that tests would soon be available to use in confirmation of the Wassermann reaction and indeed, it was predicted by some that they might some day replace it entirely.

From the inception of the Kahn Test until the present day so many precipitation tests for syphilis have been described that it is difficult to distinguish one from another. They are all, however, based on principles which are for practical purposes identical and, as has been stated by Eagle (1937), they are really only variations of one test, owing their existence to fundamentals which were established by such workers as Meinicke and Kahn. Despite the many tests which are in use at the present time, the precise nature of syphilitic antigen still remains in doubt. Precipitation tests for syphilis employ antigens made from the same materials as those used in complement fixation reactions, equally non-specific despite the "purification" mentioned above, and although much research has been carried out on the individual components of these antigens, reference to which will be made in a later section (Page 94), the exact mechanism by which an alcoholic extract of normal tissue reacts with syphilitic antibody is unknown.

In order to illustrate the investigations which follow it is proposed to give here a brief description of the technique applying to all precipitation tests for syphilis; the tests are all so similar that a general outline of the main features is sufficient.

The Antigens are almost without exception alcoholic extracts of dried animal tissue (e.g., ox heart) which has been previously extracted with acetone or ether or both. In this way only the most sensitive part of the tissue is isolated in the final antigen. (See "Chemistry of Syphilitic Antigens", Page 94 and "The Phosphatides", Page 101). Cholesterol is added to this alcoholic extract, the usual optimum being 6 mgm. per cubic centimetre of extract. To prepare the antigen for use, it is mixed with saline in stated proportions in order to produce a suspension of antigen particles which disperse finely or aggregate coarsely when brought into contact with normal or syphilitic sera respectively, under the conditions of the particular test.

The Tests are performed by placing a small quantity of this suspension, frequently 0.1 c.c. or less, in a small test-tube or on a microscope slide. A standard amount of the serum to be tested is added to the tube or slide and the mixture agitated by hand or machine for a fixed period, when the result of the test is read by naked eye or with a lens or microscope as the case may be. In most of the tests aggregation or "clumping" of the antigen particles indicates a syphilitic serum.

Tests of this nature, such as the Hinton, Kline, Mazzini, Kahn and Eagle, to mention only a few of those described, have become widely employed during the last ten years, particularly in the United States of America. They are all claimed as accurate and sensitive and in at least one well-known instance a precipitation test has completely replaced the Wassermann Reaction. (The Kahn Test in the United States Navy, Kahn, 1942.) Two of their advantages certainly cannot be disputed - (a) the ease of performance: and (b) the speed with which the tests may be conducted compared with complement fixation tests. Another feature which has an important bearing on the way in which these tests are used is the fact that their sensitivity can be increased or decreased comparatively easily, usually by simply varying the amount of saline used to transform the alcoholic extract into an emulsion of sensitive particles.

SECTION ONE

The Laughlen Reaction and the Evolution of a Standardised Modification

The Laughlen Reaction

The Laughlen Test is one of the many precipitation reactions for syphilis which have developed as a result of the original work of Meinicke and Kahn. As previously stated, these reactions are all modifications of older tests, a fact which is demonstrated with particular clarity in the Laughlen Reaction which is only a rather novel method of performing the Kahn Test. The Laughlen Reaction nevertheless possesses several features which serve to distinguish it from similar tests and to preserve its individuality as a diagnostic procedure.

First described in 1935, the outstanding feature of the test was its startling simplicity. One drop of a specially prepared antigen was placed on a microscope slide along with a drop of the serum to be tested and the two were intimately mixed by repeatedly tilting the slide by hand. A positive result was indicated by the appearance of a precipitate, the time limit for mixing being ten minutes. The test possessed something of the dramatic in that a precipitate, when it occurred, was coloured bright red due to the introduction of a dye during the preparation of the antigen and there is no doubt that this feature of the test was an attraction to many since it gave promise of being able to reduce the difficulties of the serological diagnosis of syphilis to the simplicity of an ordinary slide agglutination reaction. This naturally led to the assumption that the test could be performed by those who had little experience of serology and it is fortunate that this tendency was checked at an early period of the test's history by workers who found that although the Laughlen Test was simple to perform it required just as much attention to detail as did more intricate reactions such as the Wassermann and Kahn.

Before describing the Laughlen antigen, it is proposed to outline the preparation of Kahn antigen which is a basic constituent of the former. The following notes are taken from "The Kahn Test - A practical Guide." by R.L. Kahn, published in 1928:-

Ox heart muscle is finely ground to a paste and dried rapidly under electric fans. The dried material is now broken up and reduced to a powder by means of a coffee grinder or similar apparatus. The dry powder is now extracted with ether. Twenty-five grammes of powdered heart are extracted four times with pure anaesthetic ether, of which 100 c.c. are used for the first extraction and 75 c.c. on each of the three subsequent occasions. The ether and powder are placed together in a 250 c.c. Erlenmeyer flask and gently shaken at intervals for ten minutes, when they are separated by filtration through filter paper; any powder caught by the paper is replaced in the flask with the main part of the residue. The ether is discarded, and after the last extraction, the powder is spread out on glass to dry. When completely dry, it is weighed and placed in a clean flask and to it is added 5 c.c. of 95 per cent ethyl alcohol for every gramme of powder. The flask is shaken for ten minutes and extraction allowed to continue for three days at room temperature. The alcoholic extract is now filtered off and stored in the dark, cholesterol being added in the proportion of 6 mgm. of cholesterol per c.c. of extract. Before use, the "titre" of the antigen is estimated by making suspensions with varying amounts of physiological saline, usually 0.6, 0.8, 1.0, 1.1 and 1.2 c.c. to 1 c.c. antigen. These suspensions are left at room temperature for 30 minutes. Thereafter 0.15 c.c. saline is added to each of three separate quantities of each suspension of antigen, 0.05, 0.025, 0.0125 c.c. The mixtures, in small test-tubes, are now shaken for three minutes on the Kahn "shaker" or by hand and 0.5 c.c. saline added to each tube. Aggregation of antigen particles will be present in the tubes containing the lesser amounts of saline used to make the original suspensions, while opalescence is found in the tubes containing the greater quantities; where these two appearances meet is the titre-point of the antigen, or in other words, the "smallest amount of saline which, when added to 1 c.c. of antigen, produces aggregates capable of complete dispersion upon the addition of further saline, is the titre of the antigen".

The antigen described by Laughlen in 1935 was made as follows:- to 1 c.c. of Kahn antigen were added a small quantity of a fat stain (Scharlach R or Sudan III) and four drops of Compound Tincture of Benzoin. This mixture was warmed in a water-bath at 50° Centigrade and 10 cc. of 1.5 per cent saline, also warmed to the same temperature, was added. The pink, milky-looking fluid which resulted was known as the diluted antigen and before use it was sensitised by the addition of nine per cent salt solution, in the proportion of one drop per 1 cc. of reagent. To perform the test, one drop of this sensitised antigen was mixed on a slide with one drop of the serum under examination and gently rocked for ten minutes; precipitation occurring within this time indicated a positive result. Three years after the introduction of the test, some changes in the method of preparing the reagent were announced (Laughlen, 1938). These alterations were (a) the saline used to make the diluted antigen had now a salt content of 1.25 per cent instead of 1.5 per cent: and (b) sensitisation was performed by adding 0.15 cc. of ten per cent saline to 1 cc. of the reagent in place of the "one drop" of nine per cent saline as formerly.

As with every other "new" test for syphilis, the Laughlen Reaction was subjected to widespread trials soon after it was introduced. Many workers, especially in America, tested the reaction and compared it with other tests in order to assess its value. One of the earliest reports (Usher, 1938), stated that the test was too sensitive for routine use and emphasised the fact that its performance demanded the attention of a highly-trained staff. In the same year. it was reported (Meuther and Greutter) that the Laughlen compared well with the Kline Test but was slightly less sensitive than the Kahn, a statement, however, which was contradicted shortly afterwards (Beck, 1939) by the results of an investigation indicating that the Laughlen was equally as specific as, and more sensitive than, three well-known precipitation tests for syphilis - the Kahn, the Kolmer and the Kline. Beck's report agreed with Usher's that she had found the Laughlen required the same skilled attention as would be given to any other serological test for syphilis. A further contribution of interest was made in 1939 by Flood and Mayer who reported that they had found the Laughlen Test yielded unsatisfactory results with the sera from patients undergoing antisyphilitic treatment (vide Page 60).

Donohue (1940), stated that the Laughlen may give a negative result with a freshly-inactivated serum which at the same time produces a strongly positive Wassermann Reaction, but this statement has not been confirmed by other observers and indeed would seem to be completely contradicted by a report appearing at the same time which approved of the Laughlen as a test for use with blood-donors (Greey, 1940).

As the result of a large-scale trial carried out in 1941 the Laughlen Test was said to compare favourably with the Hinton Test (Nelson, 1941). In the same report it was emphatically affirmed that sera which were to be tested with the Laughlen must first be inactivated, a statement which directly disagreed with those of other workers including Laughlen himself, who had suggested that the test could be performed on serum without inactivation. This point is of interest in view of the findings of the present investigation (Page 48).

Several modifications of the test now began to appear without, however, altering essentially the method of making or using the antigen. Perhaps the most obvious change was that many workers now began to use the Laughlen antigen without the addition of the strong saline to effect sensitisation (Page 45). One example of this is the antigen used by Tulloch (unpublished), which is prepared from ordinary physiological saline (or saline slightly stronger), exactly as described by Laughlen but with the final addition of ten per cent saline omitted. The same procedure has been followed by Lane (1944), the only difference here being that the saline used to "dilute" the stained Kahn antigen is kept at a constant pH by means of buffer-salts in an effort to overcome the difficulty of ultrasensitiveness which develops in most Laughlen antigens when more than seven days old. One of the latest modifications of the Laughlen is that produced by Hamilton-Paterson, Cole and Usher (1944), who incorporated horse serum into the antigen with the same purpose as Lane's buffer-saline, i.e., to reduce the sensitivity. It may be presumed that the success claimed with both methods is due to the presence of buffer substances (Page 28).

Many other reports (e.g., Robinson and Stroud, 1937, Dienst and Sanderson, 1938, Craig and Calloway, 1939), testify to the reliability of the Laughlen Test, the main objection held by some workers being that the test is too sensitive and at times produces an abnormal number of non-specific positive reactions. The two modifications mentioned in the last paragraph would indicate that this over-sensitiveness may be controlled by the use of buffering agents.

The Present Modification of the Laughlen Reaction

Even in the descriptions of the most recent variations of the Laughlen Test there is a surprising lack of scientific exactness. In the making of the antigen for example, we still read of the stain being measured as a "very small quantity", "a knifepointful", etc., while the benzoin continues to be added by drops from pipettes whose calibration is doubtful. In order to produce an antigen capable of exact, scientific reproduction, it was necessary first of all to remove all doubtful methods of measurement and replace them with accurate methods since it is only these which can be duplicated at any given time. Each component of the antigen, therefore, was examined in turn in an attempt to find the best way of combining the several constituents.

Kahn Antigen:

It is a common experience in the laboratory to find that Kahn antigens from different sources vary widely in their properties. Thus, one of the most important points in preparing the Laughlen antigen is to decide which Kahn antigen will give the best results and to use that product exclusively. Using the original technique, a Laughlen antigen was made from each of four Kahn antigens, two of commercial manufacture and two prepared in the laboratory by the standard method (Kahn, 1928). Over a period of ten days tests were carried out on 276 sera using these Laughlen antigens in parallel. The results were controlled by the Wassermann (Wyler, 1929, 1932),

Table 1.

The Results Obtained with Laughlen Reagents Made From Four Different Kahn Antigens.

	Positive	Doubtful	Negative
Test No. 1. Antigens 24 Hours Old (102 Sera Tested)			
L.A. 1	28	-	74
L.A. 2	32	4	66
L.A. 3	32	16	54
L.A. 4	31	7	64
W.R.	32	11	59
Kahn	32	19	51
Test No. 2. Antigens 5 Days Old (68 Sera Tested)			
L.A. 1	7	2	59
L.A. 2	12	16	40
L.A. 3	8	3	57
L.A. 4	8	7	53
W.R.	8	1	59
Kahn	8	6	54
Test No. 3. Antigens 10 Days Old (106 Sera Tested)			
L.A. 1	14	-	92
L.A. 2	29	10	67
L.A. 3	16	5	85
L.A. 4	20	11	75
W.R.	16	2	88
Kahn	15	7	84

and Kahn Tests and are shown in Table 1. It is obvious that Laughlen antigen No. 3 was superior to the others in sensitivity and keeping quality; the Kahn antigen used here was that produced by Messrs. Burroughs Wellcome and Company and in view of the findings it was used in subsequent work. Tests with many different batches of this antigen showed that the titre, which is almost invariably 1 : 1.1 or 1 : 1.2, does not have any effect on the Laughlen reagent, presumably due to the extreme degree of dilution.

Stains:

Three stains have been described as satisfactory for the Laughlen reagent - Sudan 3, Scharlach R and Victoria Blue. Experimentally, similar results were obtained irrespective of which stain was used but the precipitation was somewhat more distinct with Scharlach R and for this reason it was employed in preference to the others. The amount of stain added to the antigen has always been somewhat indefinite and phrases such as "knifepointfuls" and "a very small quantity" occur in most descriptions of the modifications. Certainly the amount of stain required is so small that accurate measurement of the dry powder is difficult. This suggested the use of an alcoholic solution. In this way exact measurement would be possible, complete dispersion throughout the Kahn antigen would be obtained, and no undissolved particles of stain would be present in the completed antigen. As a preliminary it was necessary to find the amount of alcohol which could be added to the Kahn antigen without impairing the subsequent Laughlen antigen. Five Laughlen antigens were made from Kahn antigens to which ethyl alcohol had been added in amounts varying from 0.05 c.c. to 0.5 c.c. per 1 c.c. A Laughlen antigen prepared from the untreated Kahn antigen served as a control. Parallel tests with 400 sera over a period of four weeks showed that the addition of alcohol up to 0.15 c.c. had no apparent effect on the performance of the antigen. To determine the optimum concentration of stain varying strengths of Scharlach R from one to five per cent were prepared in absolute ethyl alcohol and added to the Kahn antigen in the selected proportion of 0.1 c.c. to 1 c.c. Tests showed that a three per cent solution of dye was best. Lower concentrations gave rise to poorly-defined precipitation and stronger solutions produced a deep red antigen in which the stain tended to form a granular deposit with negative sera. (The method of making the stain solution is referred to later.)

Effect of pH:

Several workers have drawn attention to the effect of the pH value of the saline used in precipitation tests for syphilis. Thus, Lorenz (1940), and Lane (1944), describe the use of buffered saline for the Laughlen antigen and Mazzini (1939), added buffer salts to the saline used in the test bearing his name. From the literature on the subject it would appear that the main reason for the use of these buffer-saline solutions

The work recorded herein developed from an investigation into the use of a rapid serological test for syphilis. In recent years many such tests have been investigated by laboratory workers owing to the great increase in the number of specimens sent for serological examination - an increase which has placed an immense strain on most of the laboratories dealing with serological work and particularly on public health laboratories where the volume of specimens marked "for W.R." may at times reach enormous proportions. The performance of a Wassermann Reaction on every specimen frequently becomes a laborious and almost impossible task since the test is comparatively lengthy and delicate and requires experienced supervision at every stage. The result has been a search for tests more easily and more rapidly performed than the Wassermann, capable of being carried out with simple apparatus and having a degree of sensitivity sufficient to identify all specimens giving a positive result with the complement-fixation test.

With the improvement in the technique of the many quickly-performed precipitation tests for syphilis described during the past twenty-five years, there came the hope that the serological diagnosis of this disease would become an extremely simple, swift and reliable process, and when it became increasingly obvious that precipitation tests were valuable diagnostic aids, many serologists even predicted that the Wassermann Reaction would fall into disuse. Far from this, however, the

Wassermann has become still more firmly established as the standard method of confirmation in an investigation for syphilis and even when a diagnosis of the disease has been reached by other methods few workers, either in the clinic or laboratory, would be content to omit examination of the blood by the Wassermann Reaction. This faithful adherence to the results of the complement-fixation test is not to discredit the precipitation tests but is most desirable in that it introduces a certain measure of standardisation in the methods of diagnosis.

Accordingly, it may be argued that a convenient method of diagnosing syphilis in a busy laboratory would be to examine the specimens under investigation first of all by one of the comparatively rapid precipitation tests. The sera giving negative results with this preliminary testing could be reported as such to the clinician concerned without further examination and those giving doubtful or positive reactions retained for confirmation by the Wassermann. The advantages of such a system are obvious. Much time is saved by the sorting-out or "screening" of large numbers of sera by a rapid test and also fewer specimens require to be examined by the time-consuming complement-fixation reaction. Alternatively, the initial rapid test could be carried out in small laboratories where the Wassermann might be impracticable and specimens for further testing forwarded to a larger centre.

The basis of this screening system depends on one all-important fact - the reliability of the precipitation test employed. It is essential that complete confidence can be placed in the negative results obtained with this test since these are reported without confirmation by other methods. In this instance sensitivity is vastly more important than selectivity, remembering that the purpose of the preliminary screening is to detect those sera which are definitely non-syphilitic or in other words, the sensitivity of the precipitation test must be much greater than that of the reaction, usually the Wassermann, which is used later in confirmation of doubtful and positive results.

Several precipitation reactions are now used as preliminary screen tests. The best known in this country is probably the Presumptive Kahn Test and in America quite a number have come into use in the last few years, e.g., the Kline Test, the Hinton, the Mazzini and others, including, of course, the Presumptive Kahn. In 1935, Laughlen, in Canada, described a rapid precipitation test which may be placed in the same category. Fundamentally, this reaction can be considered as a modified Kahn Test, since Kahn antigen was the basic constituent of the Laughlen antigen, but it was performed on slides in place of tubes and a novel innovation was the incorporation of a dye which rendered the precipitated particles more easily seen. Many reports on the Laughlen Test have been made by workers both in America and Britain and it is evident that the reaction has been found by the majority to be a reliable aid in the diagnosis of syphilis, its most notable features being simplicity of performance and the rapidity with which large number of sera can be tested. Several modifications of the test have been described without altering its essential structure.

The original purpose of the present investigation was to determine the suitability of the Laughlen Test for the routine examination of ante-natal specimens. As a result of the enquiry a new modification of the reaction was produced and subjected to extensive trials which indicated that this version of the test possessed high levels of sensitivity and accuracy under certain conditions. The speed of the test's performance made it an excellent method of conducting research into problems concerning syphilis antigens and this feature was made use of in the other work described in this thesis.

Some time before the completion of the investigation into the use of the Laughlen reaction as a screen test, the possibility of extracting antigenic substances from materials other than the customary heart muscle was considered: the particular substances contemplated were nervous tissue and certain plants, since the former is known to be a rich source of the lipoids forming the active portion of heart-muscle antigens and appreciable quantities of lipoids are present in vegetables. One drawback to this projected research was the length of time necessary if the Wassermann Reaction were to be used as the indicator of antigenic activity in extracts made from the substances mentioned above. The development of the modification of the Laughlen Test now suggested a convenient method of rapidly assessing the antigenic value of any such extracts by using them in place of the usual Kahn antigen basis of the modified Laughlen re-agent. By this method, examination of a phosphatide extracted from human brain yielded considerable information concerning the mode of action of the complex organic compounds constituting so-called syphilitic "antigen" and similarly it was established that antigens for precipitation tests for syphilis can be extracted from a vegetable which already has been put to innumerable uses - the soya bean.

The records of the various investigations are preceded by a brief review of the history of the syphilitic antigen since much of the work centres round this mysterious substance. This is followed by two sections, the first of which describes the steps leading to the present modification of the Laughlen Test; the second contains a description of how this version of the test was used as an instrument of research, i.e., in the enquiries into the antigenic activity of certain extracts made from nervous tissue and the soya bean.

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I wish to thank the directors of these laboratories, Professor W.J. Tulloch and Dr. R.D. Stuart, respectively, for the many facilities I was so generously afforded.

Historical Notes on the Development of Syphilis Antigen

Syphilis "antigen" is one of the most puzzling substances in the entire field of medical science. It is difficult to think of another instance where an antigen used in the diagnosis of a particular disease is so completely unrelated to the pathogenesis of that disease or indeed of any disease. Even the somewhat strange agglutination of the proteus bacillus by the serum of a typhus patient is not so mysterious as the reactions of the non-specific antigen for syphilis, yet this antigen provides the basis for tests which are among the most reliable in modern laboratory technique. Forty years have passed since it became known that the "antigens" used in tests for syphilis were not true antigens but there is still no clear understanding of their action in either the complement fixation or precipitation which they produce with syphilitic serum.

Until 1906 there existed no serological test for the diagnosis of syphilis. In that year Wassermann, Neisser and Brück published details of such a test based on the phenomena of haemolysis of red blood corpuscles by antisera (Bordet, 1898), and complement fixation (Bordet and Genou, 1901). Although there are good grounds for believing that the credit for first describing the use of complement fixation in a test for syphilis in the human subject by right belongs to Detre, whose work in this connection was also published in 1906, this method of testing for syphilitic infection is now universally known as the Wassermann Reaction. Complement, a natural constituent of serum, had been the subject of much scientific enquiry up to this time and two all-important facts had emerged to prepare the way for the elaboration of the Wassermann Reaction - (1) complement must be present before red blood corpuscles can be haemolysed by their specific anti-serum; and (2) this haemolytic property of complement, in addition to being destroyed by heat, is inhibited or "fixed" when the complement is subjected to the interaction of an anti-body with its specific antigen. It followed that, given a specific antigen, it was possible to test for the presence of the corresponding antibody by observing what happened to complement when it was placed in a mixture of the antigen and a substance under test. Red blood corpuscles, sensitised with their homologous antiserum, could be used as an indicator: haemolysis would indicate the presence of free complement which in turn would mean that the specific antibody had not been present; absence of haemolysis would show that the complement must have been "fixed" by the interaction of the antigen with its antibody, i.e., a positive result.

It seemed, therefore that all that was required for the production of a serological test for syphilis was a specific antigen, since all the other components necessary for a complement fixation test were already present, assuming, of course, that an antibody to syphilis was produced in the blood of an individual suffering from the disease. The identification of the treponema pallidum as the causal organism of syphilis (Schaudinn and Hoffmann, 1905) brought the hope that this specific antigen was now at hand since it was reasonable to suppose that this organism, in common with many other pathogenic microbes, could be used as an antigen for the disease it produced. In 1906, however, no reliable culture of the treponema was available and as an antigen for their test Wassermann and his co-workers resorted to what was probably considered the next best thing - an extract of the liver from a syphilitic foetus. This extract was made with water and when the new test began to produce results consistent with clinical findings, the obvious conclusion was that the water had removed from the liver the specific antigen of syphilis. Although this assumption has been proved to be fundamentally wrong it gave rise to a great volume of research on these tissue extracts and thus brought about an extremely rapid development of the test's potentialities. It is indeed fortunate that there were no pure cultures of the treponema pallidum in 1906, otherwise they would almost certainly have been used as the antigen for complement fixation tests and would have caused delay in the development of the Wassermann Reaction since it has been found (Schereschewsky, 1913, Kolmer, 1942) that as antigens they produce irregular and unreliable results.

The antigen-antibody theory of the Wassermann Reaction was destined to receive a serious setback only a few months after the test's inception. Early in 1907, a publication by Weygandt described an antigen made from normal spleen which had been used in a complement fixation test: this test had given positive results in definite cases of late syphilis. This finding was confirmed by many other workers and it soon became obvious that many normal organs could provide "antigens" for syphilis equally as effective as those derived from syphilitic tissue. That this should be so was puzzling in the extreme and the only answer to the problem was that the antigenic substance, supposed by Wassermann and his co-workers to have been present in their liver extract solely because of the syphilitic infection of that organ, must be a normal constituent of many organs of the animal body and as such could hardly be called a true antigen. The fact remained, nevertheless, that this unknown substance was capable of reacting <u>in vitro</u> with the antibody present in syphilitic blood in the same way as a true antigen, at least as far as concerned the phenomenon of complement fixation.

The first step towards discovering the real nature of this antigen-like substance was made in December, 1907, when Landsteiner, Müller and Potzl found that a satisfactory antigen for the Wassermann Reaction was provided by an alcoholic extract of guinea-pig heart and that the results obtained with its use were equivalent to those obtained with the original watery extract of syphilitic liver. This pointed to the fact that the "antigenic" principles were soluble in alcohol and provided the basis for widespread research directed towards discovering their chemical nature. During the next few years many reports on numerous antigen preparations were published until it appeared beyond all reasonable doubt that the antigenic action was associated with the normal lipoids which are present to a greater or less extent in all living tissue. Many individual lipoids were investigated in an effort to find the one responsible for the antigenic activity - lecithin (Porges and Meier, 1908), sodium taurocholate and glycocholate (Levaditi and Yamanouchi, 1907), cholesterin and vaselin (Fleischmann, 1908), oleic acid (Sachs and Altmann, 1908), and many other substances were subjected to intensive experiments to find what part, if any, they played in this peculiar non-specific reaction. It was found, however, that by far the best results were obtained when the antigen consisted of a tissue extract, i.e., a mixture of "crude" lipoids. These tissue lipoids were collectively referred to as "the lecithins" and later, more correctly, as "phospholipids" or phosphatides, further details and the chemistry of which will be discussed later in some detail (Page 101).

An important advance in the methods of using these "antigens" was made in 1910 when Browning, Cruickshank and

McKenzie announced the results of their work with extracts to which cholesterol had been added. Two antigens had been used in their experiments - alcoholic tissue extracts and alcoholic solutions of lecithin. The conclusions reached by these workers were (a) that the sensitivity of the so-called syphilitic antigens was markedly increased by the addition of cholesterol, and (b) that the best combination was lecithin and cholesterol. This sensitising effect of cholesterol was soon corroborated by the work of other investigators though the majority supported a later contention that even with this adjuvant the crude tissue extracts still produced the best results. (Sachs, 1911).

Five years had now elapsed since the introduction of the Wassermann Reaction and the mystery of the non-specific syphilitic antigen was still unsolved. The test was being improved and made more accurate almost daily but there was still no exact understanding of the process by which an alcoholic extract of a normal organ could act as an antigen to syphilis antibody in a complement fixation reaction. Certain important facts, however, had been established -

(a) Normal tissues produced antigens equally as satisfactory as those extracted from syphilitic organs;

(b) The antigens giving the best results were alcoholic tissue extracts; and

(c) The sensitivity of these antigens was greatly increased by the addition of cholesterol.

That these three principles were correct has been proved repeatedly in the period which has elapsed since they were formulated and at the present time most of the several modifications of the Wassermann Reaction employ as antigen an alcoholic tissue extract to which has been added cholesterol, the effect of which is to provide crystalline surfaces on which the antigen-antibody reaction takes place (Weil, 1941).

The subsequent history and development of syphilis antigen is closely connected with precipitation tests. Compared to the Wassermann Reaction, precipitation tests for syphilis are remarkable in that they developed slowly and did not become generally used until comparatively recent times. In some measure at least this was no doubt due to the amount of attention which was focused on complement-fixation. Nine years before the presentation of the Wassermann Reaction, Kraus (1897), working in Vienna, had published the first account of the phenomenon of precipitation which, so far as is known, had not been observed up to that time. Kraus's experiments were concerned with the microbes of cholera, typhoid and plague and he had found that when sterile filtrates from cultures of these organisms were added to their homologous antisera small floccules appeared in the mixtures. These "precipitates" he considered to be parts of the bacteria of the original cultures. Attempts were now made to apply this phenomenon to several diagnostic problems and as early as 1907, only a few months after the initial description of the Wassermann Reaction had been published, a report by Michaëlis gave details of his attempts to produce precipitation with syphilitic sera. In his experiments, Michaelis had employed a watery extract of syphilitic liver as antigen and had observed precipitates when it was added directly to syphilitic serum. This work does not seem to have been fully appreciated at the time for several years were to pass before real progress was made in the practical application of a precipitation test to the diagnosis of syphilis. Three years later, 1910, Jacobsthal reported the use of an alcoholic tissue extract as an antigen in a precipitation test he had developed and in the following year Brück and Hidaka used a similar antigen for a test in which the mixtures of serum and antigen were centrifuged after incubation. Alcoholic extract of heart was used by Hecht who published an account of his work in 1914.

Up to this time precipitation tests for syphilis had not been accepted generally as being of value compared with the Wassermann Reaction. There were several reasons for this; precipitation tests required long incubation periods, the antigens used were often unsatisfactory and the results obtained showed a level of sensitivity considerably lower than that of the complement-fixation test. About 1918, however, an increased interest seems to have developed and in the course of a few years many of the problems which had obstructed the progress of the precipitation reactions were solved. The first advance came when Sachs and Georgi (1918) published an account of a test employing cholesterolised antigen and in the following year Meinicke described the treatment of dried tissue with ether before making the alcoholic extract which was used as antigen. By this method of "purification" he claimed to remove unstable substances from the dry tissue. (Similar attempts to purify the antigens for the Wassermann Reaction had been made by Noguchi in 1909 but crude extracts had been found to give better results.) Meinicke's work provided the basis for practically every precipitation test subsequently developed and the preliminary treatment of dried tissue with an organic solvent such as ether or acetone has become an integral part of the preparation of antigens for use in these reactions (vide "Chemistry of Syphilis Antigen", page 94).

In 1922 the Kahn Test was described and with it there were introduced two features which have become essential procedures of almost all precipitation tests for syphilis. These were (a) the use of minimal quantities of antigen: and (b) agitation of the serum-antigen mixtures instead of incubation which had been practised hitherto. The results obtained with the Kahn Test were so striking that it was obvious that precipitation tests had at last reached the point where they could be put to practical use. The serological diagnosis of syphilis had entered a new phase and it was evident that tests would soon be available to use in confirmation of the Wassermann reaction and indeed, it was predicted by some that they might some day replace it entirely.

From the inception of the Kahn Test until the present day so many precipitation tests for syphilis have been described that it is difficult to distinguish one from another. They are all, however, based on principles which are for practical purposes identical and, as has been stated by Eagle (1937), they are really only variations of one test, owing their existence to fundamentals which were established by such workers as Meinicke and Kahn. Despite the many tests which are in use at the present time, the precise nature of syphilitic antigen still remains in doubt. Precipitation tests for syphilis employ antigens made from the same materials as those used in complement fixation reactions, equally non-specific despite the "purification" mentioned above, and although much research has been carried out on the individual components of these antigens, reference to which will be made in a later section (Page 94), the exact mechanism by which an alcoholic extract of normal tissue reacts with syphilitic antibody is unknown.

In order to illustrate the investigations which follow it is proposed to give here a brief description of the technique applying to all precipitation tests for syphilis; the tests are all so similar that a general outline of the main features is sufficient.

<u>The Antigens</u> are almost without exception alcoholic extracts of dried animal tissue (e.g., ox heart) which has been previously extracted with acetone or ether or both. In this way only the most sensitive part of the tissue is isolated in the final antigen. (See "Chemistry of Syphilitic Antigens", Page 94 and "The Phosphatides", Page 101). Cholesterol is added to this alcoholic extract, the usual optimum being 6 mgm. per cubic centimetre of extract. To prepare the antigen for use, it is mixed with saline in stated proportions in order to produce a suspension of antigen particles which disperse finely or aggregate coarsely when brought into contact with normal or syphilitic sera respectively, under the conditions of the particular test.

<u>The Tests</u> are performed by placing a small quantity of this suspension, frequently 0.1 c.c. or less, in a small test-tube or on a microscope slide. A standard amount of the serum to be tested is added to the tube or slide and the mixture agitated by hand or machine for a fixed period, when the result of the test is read by naked eye or with a lens or microscope as the case may be. In most of the tests aggregation or "clumping" of the antigen particles indicates a syphilitic serum.

Tests of this nature, such as the Hinton, Kline, Mazzini, Kahn and Eagle, to mention only a few of those described, have become widely employed during the last ten years, particularly in the United States of America. They are all claimed as accurate and sensitive and in at least one well-known instance a precipitation test has completely replaced the Wassermann Reaction. (The Kahn Test in the United States Navy, Kahn, 1942.) Two of their advantages certainly cannot be disputed - (a) the ease of performance: and (b) the speed with which the tests may be conducted compared with complement fixation tests. Another feature which has an important bearing on the way in which these tests are used is the fact that their sensitivity can be increased or decreased comparatively easily, usually by simply varying the amount of saline used to transform the alcoholic extract into an emulsion of sensitive particles.

SECTION ONE

The Laughlen Reaction and the Evolution of a Standardised Modification

The Laughlen Reaction

The Laughlen Test is one of the many precipitation reactions for syphilis which have developed as a result of the original work of Meinicke and Kahn. As previously stated, these reactions are all modifications of older tests, a fact which is demonstrated with particular clarity in the Laughlen Reaction which is only a rather novel method of performing the Kahn Test. The Laughlen Reaction nevertheless possesses several features which serve to distinguish it from similar tests and to preserve its individuality as a diagnostic procedure.

First described in 1935, the outstanding feature of the test was its startling simplicity. One drop of a specially prepared antigen was placed on a microscope slide along with a drop of the serum to be tested and the two were intimately mixed by repeatedly tilting the slide by hand. A positive result was indicated by the appearance of a precipitate, the time limit for mixing being ten minutes. The test possessed something of the dramatic in that a precipitate, when it occurred, was coloured bright red due to the introduction of a dye during the preparation of the antigen and there is no doubt that this feature of the test was an attraction to many since it gave promise of being able to reduce the difficulties of the serological diagnosis of syphilis to the simplicity of an ordinary slide agglutination reaction. This naturally led to the assumption that the test could be performed by those who had little experience of serology and it is fortunate that this tendency was checked at an early period of the test's history by workers who found that although the Laughlen Test was simple to perform it required just as much attention to detail as did more intricate reactions such as the Wassermann and Kahn.

Before describing the Laughlen antigen, it is proposed to outline the preparation of Kahn antigen which is a basic constituent of the former. The following notes are taken from "The Kahn Test - A practical Guide." by R.L. Kahn, published in 1928:-

Ox heart muscle is finely ground to a paste and dried rapidly under electric fans. The dried material is now broken up and reduced to a powder by means of a coffee grinder or similar apparatus. The dry powder is now extracted with ether. Twenty-five grammes of powdered heart are extracted four times with pure anaesthetic ether, of which 100 c.c. are used for the first extraction and 75 c.c. on each of the three subsequent occasions. The ether and powder are placed together in a 250 c.c. Erlenmeyer flask and gently shaken at intervals for ten minutes, when they are separated by filtration through filter paper; any powder caught by the paper is replaced in the flask with the main part of the residue. The ether is discarded, and after the last extraction, the powder is spread out on glass to dry. When completely dry, it is weighed and placed in a clean flask and to it is added 5 c.c. of 95 per cent ethyl alcohol for every gramme of powder. The flask is shaken for ten minutes and extraction allowed to continue for three days at room temperature. The alcoholic extract is now filtered off and stored in the dark, cholesterol being added in the proportion of 6 mgm. of cholesterol per c.c. of extract. Before use, the "titre" of the antigen is estimated by making suspensions with varying amounts of physiological saline, usually 0.6, 0.8, 1.0, 1.1 and 1.2 c.c. to 1 c.c. antigen. These suspensions are left at room temperature for 30 minutes. Thereafter 0.15 c.c. saline is added to each of three separate quantities of each suspension of antigen, 0.05, 0.025, 0.0125 c.c. The mixtures, in small test-tubes, are now shaken for three minutes on the Kahn "shaker" or by hand and 0.5 c.c. saline added to each tube. Aggregation of antigen particles will be present in the tubes containing the lesser amounts of saline used to make the original suspensions, while opalescence is found in the tubes containing the greater quantities: where these two appearances meet is the titre-point of the antigen, or in other words, the "smallest amount of saline which, when added to 1 c.c. of antigen, produces aggregates capable of complete dispersion upon the addition of further saline, is the titre of the antigen".

The antigen described by Laughlen in 1935 was made as follows:- to 1 c.c. of Kahn antigen were added a small quantity of a fat stain (Scharlach R or Sudan III) and four drops of Compound Tincture of Benzoin. This mixture was warmed in a water-bath at 50° Centigrade and 10 cc. of 1.5 per cent saline, also warmed to the same temperature, was added. The pink, milky-looking fluid which resulted was known as the diluted antigen and before use it was senstitised by the addition of nine per cent salt solution, in the proportion of one drop per 1 cc. of reagent. To perform the test, one drop of this sensitised antigen was mixed on a slide with one drop of the serum under examination and gently rocked for ten minutes; precipitation occurring within this time indicated a positive result. Three years after the introduction of the test, some changes in the method of preparing the reagent were announced (Laughlen, 1938). These alterations were (a) the saline used to make the diluted antigen had now a salt content of 1.25 per cent instead of 1.5 per cent: and (b) sensitisation was performed by adding 0.15 cc. of ten per cent saline to 1 cc. of the reagent in place of the "one drop" of nine per cent saline as formerly.

As with every other "new" test for syphilis, the Laughlen Reaction was subjected to widespread trials soon after it was introduced. Many workers, especially in America, tested the reaction and compared it with other tests in order to assess its value. One of the earliest reports (Usher, 1938), stated that the test was too sensitive for routine use and emphasised the fact that its performance demanded the attention of a highly-trained staff. In the same year. it was reported (Meuther and Greutter) that the Laughlen compared well with the Kline Test but was slightly less sensitive than the Kahn, a statement, however, which was contradicted shortly afterwards (Beck, 1939) by the results of an investigation indicating that the Laughlen was equally as specific as, and more sensitive than, three well-known precipitation tests for syphilis - the Kahn, the Kolmer and the Kline. Beck's report agreed with Usher's that she had found the Laughlen required the same skilled attention as would be given to any other serological test for syphilis. A further contribution of interest was made in 1939 by Flood and Mayer who reported that they had found the Laughlen Test yielded unsatisfactory results with the sera from patients undergoing antisyphilitic treatment (vide Page 60).

Donohue (1940), stated that the Laughlen may give a negative result with a freshly-inactivated serum which at the same time produces a strongly positive Wassermann Reaction, but this statement has not been confirmed by other observers and indeed would seem to be completely contradicted by a report appearing at the same time which approved of the Laughlen as a test for use with blood-donors (Greey, 1940).

As the result of a large-scale trial carried out in 1941 the Laughlen Test was said to compare favourably with the Hinton Test (Nelson, 1941). In the same report it was emphatically affirmed that sera which were to be tested with the Laughlen must first be inactivated, a statement which directly disagreed with those of other workers including Laughlen himself, who had suggested that the test could be performed on serum without inactivation. This point is of interest in view of the findings of the present investigation (Page 48).

Several modifications of the test now began to appear without, however, altering essentially the method of making or using the antigen. Perhaps the most obvious change was that many workers now began to use the Laughlen antigen without the addition of the strong saline to effect sensitisation (Page 45). One example of this is the antigen used by Tulloch (unpublished), which is prepared from ordinary physiological saline (or saline slightly stronger), exactly as described by Laughlen but with the final addition of ten per cent saline omitted. The same procedure has been followed by Lane (1944), the only difference here being that the saline used to "dilute" the stained Kahn antigen is kept at a constant pH by means of buffer-salts in an effort to overcome the difficulty of ultrasensitiveness which develops in most Laughlen antigens when more than seven days old. One of the latest modifications of the Laughlen is that produced by Hamilton-Paterson, Cole and Usher (1944), who incorporated horse serum into the antigen with the same purpose as Lane's buffer-saline, i.e., to reduce the sensitivity. It may be presumed that the success claimed with both methods is due to the presence of buffer substances (Page 28).

Many other reports (e.g., Robinson and Stroud, 1937, Dienst and Sanderson, 1938, Craig and Calloway, 1939), testify to the reliability of the Laughlen Test, the main objection held by some workers being that the test is too sensitive and at times produces an abnormal number of non-specific positive reactions. The two modifications mentioned in the last paragraph would indicate that this over-sensitiveness may be controlled by the use of buffering agents.

The Present Modification of the Laughlen Reaction

Even in the descriptions of the most recent variations of the Laughlen Test there is a surprising lack of scientific exactness. In the making of the antigen for example, we still read of the stain being measured as a "very small quantity","a knifepointful", etc., while the benzoin continues to be added by drops from pipettes whose calibration is doubtful. In order to produce an antigen capable of exact, scientific reproduction, it was necessary first of all to remove all doubtful methods of measurement and replace them with accurate methods since it is only these which can be duplicated at any given time. Each component of the antigen, therefore, was examined in turn in an attempt to find the best way of combining the several constituents.

Kahn Antigen:

It is a common experience in the laboratory to find that Kahn antigens from different sources vary widely in their properties. Thus, one of the most important points in preparing the Laughlen antigen is to decide which Kahn antigen will give the best results and to use that product exclusively. Using the original technique, a Laughlen antigen was made from each of four Kahn antigens, two of commercial manufacture and two prepared in the laboratory by the standard method (Kahn, 1928). Over a period of ten days tests were carried out on 276 sera using these Laughlen antigens in parallel. The results were controlled by the Wassermann (Wyler, 1929, 1932),

Table 1.

The Results Obtained with Laughlen Reagents Made From Four Different Kahn Antigens.

	Positive	Doubtful	Negative
Test No. 1. Antigens 24 Hours Old (102 Sera Tested)			
L.A. 1	28	-	74
L.A. 2	32	4	66
L.A. 3	32	16	54
L.A. 4	31	7	64
W.R.	32	11	59
Kahn	32	19	51
Test No. 2. Antigens 5 Days Old (68 Sera Tested)			
L.A. 1	7	2	59
L.A. 2	12	16	40
L.A. 3	8	3	57
L.A. 4	8	7	53
W.R.	8	1	59
Kahn	8	6	54
Test No. 3. Antigens 10 Days Old (106 Sera Tested)			
L.A. 1	14	-	92
L.A. 2	29	10	67
L.A. 3	16	5	85
L.A. 4	20	11	75
W.R.	16	2	88
Kahn	15	7	84

and Kahn Tests and are shown in Table 1. It is obvious that Laughlen antigen No. 3 was superior to the others in sensitivity and keeping quality; the Kahn antigen used here was that produced by Messrs. Burroughs Wellcome and Company and in view of the findings it was used in subsequent work. Tests with many different batches of this antigen showed that the titre, which is almost invariably 1 : 1.1 or 1 : 1.2, does not have any effect on the Laughlen reagent, presumably due to the extreme degree of dilution.

Stains:

Three stains have been described as satisfactory for the Laughlen reagent - Sudan 3, Scharlach R and Victoria Blue. Experimentally, similar results were obtained irrespective of which stain was used but the precipitation was somewhat more distinct with Scharlach R and for this reason it was employed in preference to the others. The amount of stain added to the antigen has always been somewhat indefinite and phrases such as "knifepointfuls" and "a very small quantity" occur in most descriptions of the modifications. Certainly the amount of stain required is so small that accurate measurement of the dry powder is difficult. This suggested the use of an alcoholic solution. In this way exact measurement would be possible, complete dispersion throughout the Kahn antigen would be obtained, and no undissolved particles of stain would be present in the completed antigen. As a preliminary it was necessary to find the amount of alcohol which could be added to the Kahn antigen without impairing the subsequent Laughlen antigen. Five Laughlen antigens were made from Kahn antigens to which ethyl alcohol had been added in amounts varying from 0.05 c.c. to 0.5 c.c. per 1 c.c. A Laughlen antigen prepared from the untreated Kahn antigen served as a control. Parallel tests with 400 sera over a period of four weeks showed that the addition of alcohol up to 0.15 c.c. had no apparent effect on the performance of the antigen. To determine the optimum concentration of stain varying strengths of Scharlach R from one to five per cent were prepared in absolute ethyl alcohol and added to the Kahn antigen in the selected proportion of 0.1 c.c. to 1 c.c. Tests showed that a three per cent solution of dye was best. Lower concentrations gave rise to poorly-defined precipitation and stronger solutions produced a deep red antigen in which the stain tended to form a granular deposit with negative sera. (The method of making the stain solution is referred to later.)

Effect of pH:

Several workers have drawn attention to the effect of the pH value of the saline used in precipitation tests for syphilis. Thus, Lorenz (1940), and Lane (1944), describe the use of buffered saline for the Laughlen antigen and Mazzini (1939), added buffer salts to the saline used in the test bearing his name. From the literature on the subject it would appear that the main reason for the use of these buffer-saline solutions

Table 2.

The Effect of Glassware on the Sensitivity of the Laughlen Reagent.

(a) First Testing (100 Unselected Sera)

	Strongly Positive	Doubtful	Negative
Antigen No. 1	16	5	79
Antigen No. 2	16	8	76
Wassermann	16	2	82

(b) Second Testing (100 Unselected Sera)

	Strongly Positive	Doubtful	Negative
Antigen No. 1	36	18	46
Antigen No. 2	22	3	75
Wassermann	21	1	78

The second testing was carried out three weeks after the first, i.e., when the antigens were 21 days old.

Antigen No. 1 made with 1.5 per cent saline in ordinary test-tubes.

Antigen No. 2 made with 1.5 per cent saline in Pyrex glass test-tubes.

is to control over-sensitivity and to eliminate false positive reactions. In connection with the latter, Hamilton-Paterson, Cole and Usher (1944), in their modification of the Laughlen, employ horse serum which presumably exerts a buffering action in much the same way as would a combination of buffer salts.

Before testing the effect of buffered saline in the present investigation, a simple experiment was carried out to assess the influence of ordinary soda glass on the completed antigen. It is well known that soda glass tends to make contained solutions more alkaline and if this effect was exerted on the diluting saline for the Laughlen antigen it would be impossible to investigate accurately the changes brought about by controlled variations in the pH value of the saline. Table 2 shows how this test was carried out. Two Laughlen antigens were made, one in ordinary test-tubes, and the other in Pyrex tubes according to the original formula (Page 21) with the exception that the stain was added as described in the previous paragraph. These antigens were tested when two days old and again when three weeks old, 100 unselected sera being used on each occasion and from the results in Table 2 there is no doubt that the antigen made in ordinary test-tubes developed marked oversensitiveness within three weeks of manufacture. When physiological saline, pH 6.8, was kept in a soda glass-tube for three weeks the pH value rose to about 8, but when kept in Pyrex test-tubes no such change was observed. From this it

Table 3.

The Effect of pH on the Laughlen Reagent

pH of Saline Used to Dilute Antigen	Strongly Positive	Doubtful	Negative
5	33	6	261
5.5	39	13	248
6	41	13	246
6.5	41	12	247
7	46	24	230
7.5	47	32	221
8	59	39	202
Wassermann Reaction	39	5	256
Kahn Reaction	41	11	248

The results obtained with seven Laughlen antigens made with 1.5 per cent saline of pH varying from five to eight. Three hundred unselected sera employed, tested in parallel with the Wassermann and Kahn Reactions.

was concluded that if the pH value of the diluting saline for the Laughlen antigen could be maintained at a constant figure the development of ultrasensitivity might be considerably delayed and the antigen thus be capable of producing more constant results. The optimum pH value was now sought. It was presumed that this would lie somewhere near neutrality and thus, using Sörensen's phosphate buffers, buffer-solutions containing 1.5 per cent NaCl were made up with pH values ranging from 5 to 8. Laughlen antigens were prepared using these solutions as diluting saline and, after 48 hours, were tested with 300 unselected sera in parallel with Wassermann and Kahn Tests. The results are shown in Table 3, and it is obvious that the Laughlen agrees closely with the findings of the Wassermann and Kahn reactions when the pH of the diluting saline is about 6.5. The antigens were now left until 14 days old and tested again in a similar manner. The results of this second test were almost identical with those of the first, while a closer investigation of the pH figure revealed that the optimum value was 6.6.

<u>Concentration of NaCl</u>:

So far the diluting saline had been used at the strength originally employed by Laughlen, i.e., 1.5 per cent, though a later publication (Laughlen, 1938) suggested that better results were obtained when this concentration is reduced to 1.25 per cent. It was now necessary to determine the optimum

Table 4.

The Effect of Variation of the NaCl Content of the Diluting Saline on the Sensitivity of the Modified Laughlen Antigen.

NaCl Concentration:	Strongly Positive	Doubtful	Negative
0.5%	132	19	349
0.7%	160	27	313
0.9%	164	36	300
1.0%	164	52	284
1.25%	166	51	283
1.5%	170	60	270
3.0%	197	68	235
Wassermann	157	21	322
Kahn	162	33	305

Tests conducted, in parallel with the Wassermann and Kahn reactions, on 500 unselected sera from patients attending Venereal Disease Clinics.

salt content with the diluent buffered at the fixed pH of 6.6. Seven solutions, buffered at this level, were made up containing respectively 0.5 per cent, 0.7 per cent 0.9 per cent, 1 per cent, 1.25 per cent, 1.5 per cent and 3 per cent NaCl, and using them as diluting saline, seven Laughlen reagents were made. Tests were made with these antigens, in parallel with the Wassermann and Kahn Reactions, in a series of 500 sera taken from patients at their first attendance at a Venereal Disease Clinic, i.e., "diagnostic" specimens. The results are shown in Table 4 from which it seems quite definite that the optimum concentration of NaCl is 0.9 per cent, using the Wassermann Reaction and Kahn as standards of comparison.

One striking difference was noted when working with buffered saline. When no buffer salts are present, the sensitivity of the antigen could be altered quite sharply by a small change in salt content but with buffered saline the alteration was more gradual and could be obtained only with wide differences in NaCl content. This might indicate that the phospholipid particles of the original extract (Kahn antigen) are first sensitised by the salt and that thereafter the sensitivity of the antigen depends of the pH of the surrounding medium.

Amount of Benzoin:

In the preceding experiments, the Compound of Tincture of Benzoin had been added to the antigen by "drops" as described

Table 5.

Variation in the Sensitivity of Laughlen Antigen Caused by Altering the Tr. Benz. Co. Content

Amount of Benzoin	Strongly Positive	Doubtful	Negative
0.05 cc.	All tests granular - unreadable		
0.10 cc.	42	26	32
0.15 cc.	36	16	48
0.20 cc.	28	10	62
0.25 cc.	27	10	63
0.30 cc.	23	7	70
0.35 cc.	18	4	78
0.40 cc.	18	5	77
0.45 cc.	13	4	83
0.50 cc.	13	-	87
Wassermann	20	4	76
Kahn	20	10	70

One hundred selected sera examined in parallel with the Wassermann and Kahn Tests.

in the original publication. Several of the latest modifications (Lane, 1944, Hamilton-Paterson et al., 1944) have the benzoin added from a calibrated "dropping" pipette in an effort to overcome the disadvantage of inaccurate measurement, but even with dropping pipettes difficulties may arise - the slightest injury to the tip of the pipette, for example, is enough to nullify all attempts at accuracy. There does not seem to be any reason why this component should not be added to the other constituents by means of an ordinary graduated pipette: this would make its measurement completely standard and avoid the necessity for the somewhat lengthy procedure of calibrating special "droppers".

Ten Laughlen antigens were prepared, incorporating the modifications described in previous paragraphs, and containing Tr. Benz. Co. in amounts between 0.05 cc. and 0.5 cc. per 1 cc. of Kahn antigen, increasing by 0.05 cc. quantities - an ordinary 1 cc. calibrated pipette was used for these measurements. Tests were carried out with these antigens using 100 selected sera - the results are shown in Table 5. It will be seen that the 0.3 cc. amount produced the best results: amounts greater than this gave increasing stability, while the smaller quantities produced "watery" antigens which were over-sensitive. It is obvious that the sensitivity of the Laughlen antigen can be altered by variation of the benzoin content and it may be that this would prove to be a better way of controlling sensitivity than the more usual methods of altering the NaCl concentration. This observation agrees with a finding by Laughlen (1935), that it is the colloidal nature of the Laughlen antigen which prevents the complete aggregation of the antigen particles, the colloidal state being due to the benzoin present. Presumably, then, the density of the colloid is in direct proportion to the degree of agglutination which may be produced, or in other words, to the degree of sensitivity of the antigen

One important practical point is that the Tr. Benz. Co. used in the preparation of the antigen must be made with absolute ethyl alcohol. Owing to present-day restrictions, methyl alcohol is frequently substituted, and when this has been done most irregular results are obtained with the completed antigen. Presumably there is some reaction between the methyl alcohol and the ethyl alcohol of the Kahn antigen and stain solution, leading to impairment of the final reagent.

Temperature:

Experiments were made to discover if the temperature of the water-bath (in which the diluting saline and the modified Kahn antigen are warmed) is in fact optimum at the level stated by Laughlen and subsequently used in all modifications. Table 6 shows the results which were obtained with antigens made at temperatures ranging from 22°Centigrade to 70°

Table 6.

The Effect of Variations in the Temperature at Which the Antigen is Made.

Serum	W.R.	Kahn	Temperature in Degrees Centigrade							
			22	33	37	45	50	55	60	70
1	-	-	-	-	-	-	-	-	+	All tests granular - unreadable.
2	-	-	-	-	-	-	-	-	-	
3	-	-	-	-	-	-	-	-	+	
4	-	-	-	-	-	-	-	+	++	
5	-	-	-	-	-	-	-	-	-	
6	-	-	-	-	-	-	-	-	+	
7	-	-	-	-	-	-	-	+	+	
8	-	-	-	-	-	-	-	-	+	
9	-	-	-	-	-	-	-	-	-	
10	-	-	-	-	-	-	-	-	++	
11	++	++	-	+	+	++	+++	+++	+++	
12	++	++	+	+	++	++	+++	+++	++++	
13	++	++	-	+	+	++	+++	+++	+++	
14	++	++	+	+	++	++	++	+++	+++	
15	++	+++	+	+	++	++	+++	+++	+++	
16	++	+++	++	++	++	++	+++	+++	++++	
17	++	+++	+	+	++	++	+++	++++	++++	
18	++	++++	++	++	++	+++	++++	++++	++++	
19	++	++++	+++	+++	+++	+++	++++	++++	++++	
20	++	++++	++	++	+++	+++	++++	++++	++++	

Twenty selected sera tested in parallel with W.R. and Kahn.

Centigrade and there is no doubt that the level used by Laughlen, i.e., 50° Centigrade, is by far the best at which to prepare the antigen. This temperature is in fact quite critical and a deviation of as little as 3° Centigrade is enough to produce changes in the antigen which becomes more stable or more sensitive when prepared at temperatures below or above 50° Centigrade, respectively.

<u>Final Sensitisation of the Antigen with Strong Saline</u>:

It will be remembered (Page 24) that in most of the recent modifications of the Laughlen Test, this sensitisation has been omitted. The need for it apparently depends on the sensitivity of the Kahn antigen used and it was found during the present investigation that a Laughlen reagent made from a Kahn antigen of known low sensitivity (e.g., the "L.A.1" in Table 1) could be made to assume a sensitivity equal to that of a reagent made from the selected Kahn antigen (Burroughs Welcome) by the addition of varying amounts of ten per cent saline. In another modification (Lane, 1944), using "Presumptive" Kahn Antigen - an antigen more sensitive than the ordinary product - the sensitisation by strong saline is not employed. A recent communication from Professor Tulloch of Dundee confirms these findings, and certainly in the present modification, using the Kahn antigen described, the final sensitisation by nine per cent or ten per cent salt solution has been found to be unnecessary (vide Page 45).

All the components of the Laughlen antigen had now been examined and exact methods of measurement established. It remained to incorporate these findings into the production of the final modification and by testing this modified version of the reagent against a large number of sera in parallel with the Wassermann and Kahn Tests, assess its value in the diagnosis of syphilis.

Preparation of the Modified Antigen

Apparatus and Reagents:

(1) Kahn Antigen - Burroughs Wellcome and Company (Page 26)

(2) Stain - As described on Page 28, this is made up as a three per cent solution, Scharlach R being the selected stain. To 100 cc. of absolute ethyl alcohol in a 250 cc. flask is added 3 gm. of Scharlach R stain powder. The flask, tightly stoppered with cotton wool covered with tin foil, is shaken on a Kahn "shaker" for five minutes and then placed in an incubator at 37° Centigrade for thirty minutes. The resulting solution is dark red and contains a thick deposit of insoluble material which is removed by filtering twice through Whatman's No. 1 filter paper. The volume is made up to 100 cc. with absolute alcohol and the preparation is stored in a glass-stoppered bottle in the dark.

(3) Buffer-Saline - Sørensen's M/15 solutions were found to be convenient for this purpose. These are two phosphate

mixtures which are made by dissolving 9.08 gm. potassium acid phosphate (KH_2PO_4) and 9.47 gm. anhydrous sodium phosphate (Na_2HPO_4) each in one litre of water. Combinations of varying amounts of these solutions produce buffered mixtures at preselected pH values and to make one litre of solution at pH 6.6 (the optimum value for the diluting saline), 625 cc. of the potassium mixture is added to 375 cc. of the sodium mixture. (Stitt, 1943). To this is added 9 gm. of NaCl, to give the required concentration of sodium chloride (0.9 per cent). The pH value tends to be lowered to 6.5 with the addition of the NaCl but this has been found to be of no consequence.

(4) Compound Tincture of Benzoin - A fairly large quantity should be obtained in order to ensure that the same tincture is used each time the antigen is made up. As noted on Page 33 care should be taken to ensure that absolute ethyl alcohol is used in the preparation of the tincture.

(5) Water-Bath - accurately controlled at 50° Centigrade.

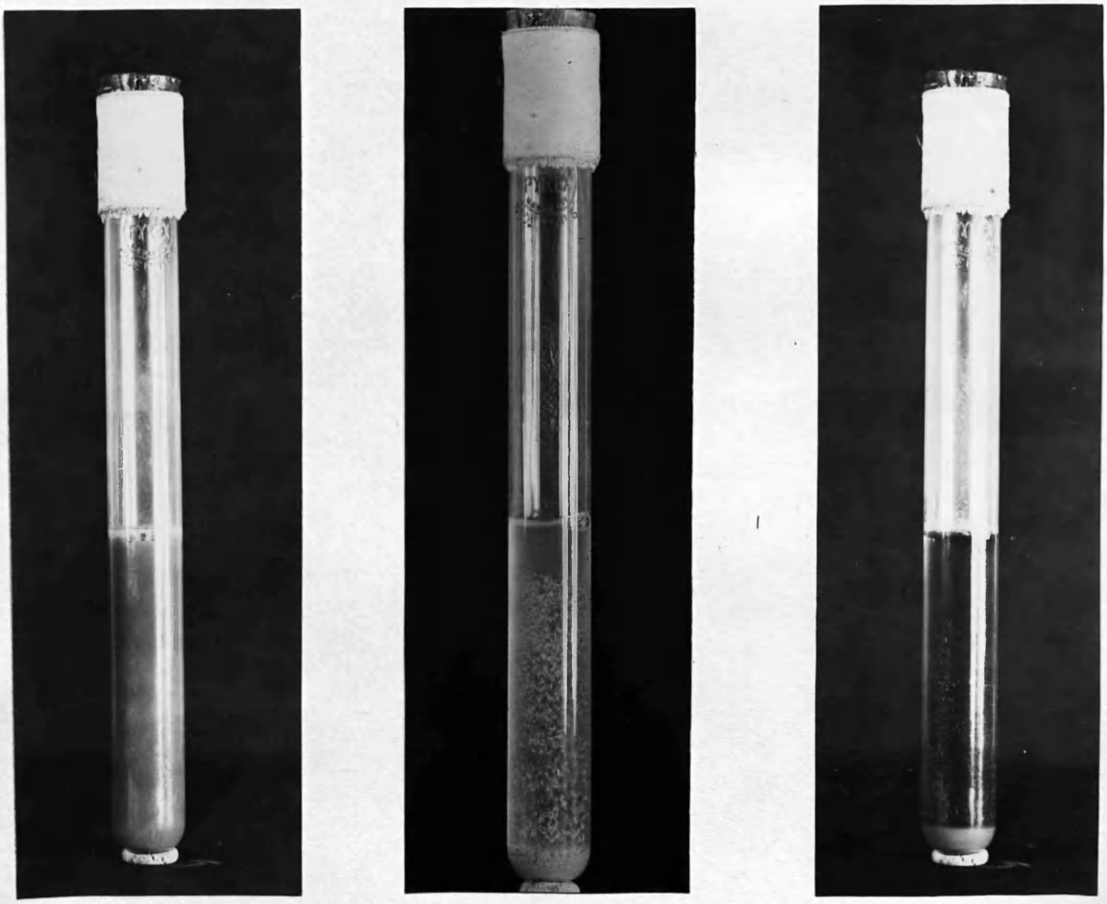
(6) Calibrated Pipettes - 1 cc. and 10 cc.

(7) Pyrex Test-Tubes - 6" X $\frac{5}{8}$".

Making the Antigen:

Into one of the Pyrex tubes is pipetted exactly 10 cc. of the buffer-saline. This tube is placed upright in the water-bath and left in this position while the other components of the antigen are prepared. To 1 cc. of the Kahn antigen in a

Figure 1.



(a) (b) (c)

Three Stages in the Sedimentation of the Modified Antigen

(a) Newly made

(b) After standing 12 hours

(c) After standing 30 hours

second Pyrex tube is added 0.1 cc. of the three per cent stain solution, the tube being shaken for a few seconds to ensure complete mixing; 0.3 cc. of the Tr. Benz. Co. is now added and this second tube placed in the water bath for exactly two minutes. Both tubes are then lifted out and their contents mixed by pouring the saline into the Kahn antigen-stain-benzoin mixture then back and forwards four times. The resulting pink liquid is the completed modified Laughlen antigen. It has the appearance of a colloidal suspension and may be described as looking like "pink milk". Within a few hours it begins to sediment and eventually becomes a pale pink fluid with a reddish deposit (Figure 1). This sedimentation does not impair the qualities of the antigen; before use the colloidal appearance is restored by inverting the container several times. The tube containing the antigen is stoppered with an ordinary cork, dated, and stored in the dark (vide Page 46).

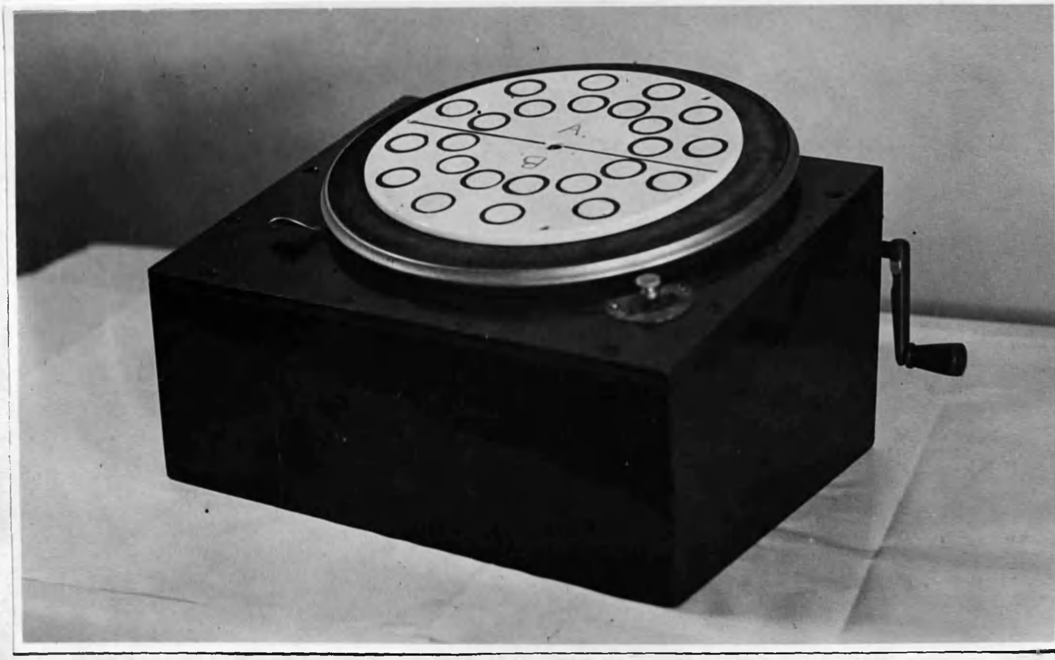
A series of tests was now carried out with this modified antigen to discover if it produced results in any way comparable to other tests for syphilis and to assess its behaviour under a variety of conditions. The apparatus used is first described.

The Apparatus and the Method Of Performing The Test

As has been described (Page 15) agitation of the mixture of antigen and serum is an essential part of the precipitation tests for syphilis. Each test has its own method of performing this agitation and the procedures vary from the simple rocking by hand of a microscope slide to the elaborate shaking machines used, for example, in the Kahn Test. Slides were used in the original Laughlen Reaction but when examining a large number of sera a great deal of time is saved if the tests are performed simultaneously. For this purpose Laughlen suggested the use of large glass plates, marked off into sections, but it has been the experience in the present investigation that the various degrees of precipitation are more easily seen when a porcelain surface is used in place of glass and that a white "tile", such as is used for blood-grouping, answers the purpose excellently. Separation of the individual tests can be accomplished by placing each mixture of antigen and serum within a circle painted on the surface of the tile.

Some mechanical method of agitation is desirable when dealing with large numbers and a machine capable of giving a rotary movement to a tilted tile is required in order to imitate as closely as possible the mixing obtained by hand-agitation. A simple and effective way of doing this is to use a circular white porcelain plate about 10" in diameter with a central hole large enough to accommodate a gramophone record

Figure 2.



The Improvised automatic Mixer

Showing one of the Marked Plates in Position on the Turntable.

spindle. When this plate is placed on the turntable of a gramaphone which has been tilted by raising one side on a small block of wood, the requisite movement is obtained when the motor is started. A more permanent machine can be made by removing the motor from its cabinet and building it into a box with angled sides - the turntable is thus kept in a constant inclined plane. Figure 2 shows one such model which has been found satisfactory. The only mechanical alteration required concerns the speed control which is modified to obtain the fairly slow rate of 18 revolutions per minute. In most makes of gramophone this is quite simply done by making a slight bend in the arm connecting the manual speed control to the motor.

A porcelain plate of the size described is large enough to accommodate 24 of the painted circles. As shown in the photograph this is done by dividing the plate into two equal parts by a line running through the centre. In each half-section are painted 12 one-inch circles in two semicircular rows of six. The simplest method of marking the circles is to use the lid of a one-ounce screw-capped bottle (H53.U.G.B.) as a marker by dipping its rim into black enamel and "stamping" the outline on the surface of the plate. When the enamel has dried, the process is repeated in order to build up a "wall" round each circular area. For rapid work it is best to have two of these marked plates.

The only other apparatus required are two pipettes. The selection and calibration of these will be described in the section dealing with the proportion of antigen to serum (Page 43).

<u>Method of Performing the Test</u>:

Racks to hold the tubes of serum are designed to take 12 specimens per row; it is convenient to have racks which can accommodate two or more rows, each of which is given a reference letter - A. B. etc.

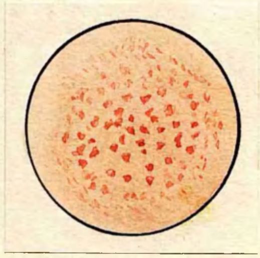
The operator sits with a porcelain plate before him on the bench with a rack of specimens immediately beyond. The reference letter of the row to be tested is grease-pencilled on the plate as shown in Figure 2. Using the appropriate pipette, which is rinsed out once with saline between specimens, one drop of each serum is placed in its respective circle, working from left to right, and starting with the outer six circles. (The fact that some specimens are further away from the centre of the plate and consequently receive a greater "throw" during rotary mixing than those on the inner portion does not affect the final result: this was confirmed by testing over 200 selected sera giving all grades of precipitation.)

With one row completed the plate is turned so that the other half is presented to the operator and the next row is pipetted. Twenty-four sera are now represented on the surface of the plate; to each specimen is added one drop of the antigen which has been gently shaken or mixed by inversion before use. The motor of the "shaker" is wound up and the plate placed on the turntable which is set going at the standard speed of 18 - 20 revolutions per minute for a period of ten minutes. This "standard speed" is somewhat empirical and is simply the number of revolutions per minute which makes for smooth mixing of the contents of the circles without being fast enough to cause overflowing. The mixture of antigen and serum should fill each circle and flow round smoothly inside its margin but if any remains as a blob of liquid the motor should be stopped momentarily and the fluid spread out gently with a fine piece of wire to fill the circle's area. When five minutes have elapsed the next two rows can be prepared on a second plate - with experience this takes almost exactly five minutes so that the first plate is due to be read when the second is ready for the turntable. From then on the first five minutes of every ten-minute period is employed in reading, washing and drying the last plate to be taken off the turntable. This ten-minute period has been used with most versions of the Laughlen Test and has been found to be the most convenient time for antigen and serum to be in contact before reading the result. Any precipitation which is going to appear will do so within six to seven minutes; the extra three minutes serve to intensify a weakly positive reaction.

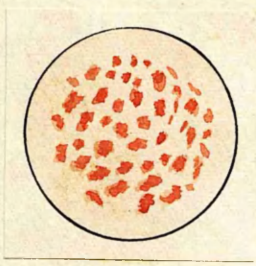
To read the test, the plate is laid flat on the bench and

Figure 3.

POSITIVE + +



POSITIVE + + +



POSITIVE + + + +

The Degrees of Precipitation. ++. +++. ++++

the contents of each circle examined, using a x6 hand lens. The following are the appearances which may be found -

(a) The mixture remains uniformly colloidal. No precipitate is visible even with the lens. NEGATIVE.

(b) A very faint precipitate visible with the lens. DOUBTFUL.+

(c) A definite fine precipitate, visible to the naked eye. POSITIVE + +.

(d) A heavy precipitate, consisting of approximately 30 - 40 particles. POSITIVE + + +.

(e) A very heavy precipitate, concentrated into 12 or less "clumps". POSITIVE + + + +.

These five appearances are discussed on Page 57 and the positive grades of precipitation are shown diagramatically in Figure 3. The test is best read under a fairly bright artificial light, a microscope lamp being convenient for the purpose, but it is emphasised that some little experience is required before all the degrees of precipitation can be diagnosed with speed and certainty.

Table 7.

The Reactions of Fixed Amounts (0.05 cc.) of Syphilitic Sera with Varying Quantities of Antigen.

Amount of Antigen	Degree of Precipitation			
	Serum 1	Serum 2	Serum 3	Serum 4
0.01 0.015 0.02 0.025	With these amounts of antigen the degree of precipitation was so fine as to be practically unreadable.			
0.03	++	++	+	+
0.035	+++	++	++	++
0.04	++++	++++	+++	++
0.045	++++	++++	+++	+++
0.05	++++	+++(+)	+++	+++
0.055	++++	+++	+++	++(+)
0.06	+++	+++	++(+)	++
0.065	+++	++(+)	++	++
0.07	+++	++	++	++
0.075	++(+)	++	++	+(+)
0.08	++	++	+(+)	+(+)
0.085 0.09 0.095 0.1	With these amounts of antigen the precipitates, though definite, were too heavily "masked" by excess fluid to be clearly readable.			
Wassermann	++	++	++	+
Kahn	++++	+++	++	++

The brackets ( ) indicate "not quite", i.e., +(+) means that the reaction was not strong enough to be classed as ++ but was definitely better than + .

The Properties of the Modified Antigen

The reactions of the antigen under certain conditions were now studied and the results grouped under the following headings -

(a) The proportions of antigen and serum.

(b) Sensitivity.

(c) Sensitisation with ten per cent saline.

(d) Conditions of storage.

(e) Bile-stained sera.

(f) Examination of plasma.

(g) Inactivation of sera.

(h) Testing the modified antigen over a large series.

(i) The effect of pregnancy.

(j) The effect of carcinoma.

(k) The effect of malaria

(l) The effect of tuberculosis.

(a) The Proportions of Antigen and Serum:

In the original Laughlen Test the antigen:serum ratio was approximately 1 : 1 and both components were measured in "drops" from presumably similar pipettes, a procedure which has been followed by most of the subsequent modifications. The optimum ratio for the present antigen was sought by testing it in varying proportion against several sera of known reactivity to the Wassermann and Kahn Tests (Table 7). The amount of serum was kept constant at 0.05 cc. and the antigen

Table 8.

The Effect of Aging on the Sensitivity of the Modified Antigen.

	One Hour			Six Hours			Twelve Hours			Twenty-Four Hours			Thirty Hours			Thirty-Six Hours			Forty-Eight Hours		
	*++	±	-	++	±	-	++	±	-	++	±	-	++	±	-	++	±	-	++	±	-
Wassermann	13	5	32	13	5	32	13	5	32	13	5	32	13	5	32	13	5	32	13	5	32
Kahn	13	7	30	13	7	30	13	7	30	13	7	30	13	7	30	13	7	30	13	7	30
M.L.1	6	-	44	9	3	38	11	6	33	13	6	31	13	6	31	13	7	30	13	7	30
M.L.2	6	2	42	10	2	38	12	-	38	13	6	31	13	7	30	14	6	30	14	6	30
M.L.3	4	2	44	9	2	39	11	7	32	13	4	33	13	7	30	14	6	30	14	6	30
M.L.4	6	-	44	9	3	38	12	6	32	13	6	31	13	7	30	13	7	30	13	7	30

(The same 50 sera were used during the first 48 hours of testing.)

	Four Days			Ten Days			Twenty-One Days			Two Months			Three Months		
	++	±	-	++	±	-	++	±	-	++	±	-	++	±	-
Wassermann	6	1	43	15	3	32	11	3	36	6	-	44	9	2	39
Kahn	6	-	44	17	1	32	11	1	38	6	-	44	9	-	41
M.L.1	6	4	40	17	-	33	11	6	33	6	-	44	11	7	32
M.L.2	6	3	41	17	4	29	12	3	35	6	-	44	11	4	35
M.L.3	6	3	41	17	1	32	12	3	35	6	-	44	10	5	35
M.L.4	6	2	42	17	-	33	11	6	33	6	-	44	11	7	32

* ± Includes the + M.L. precipitation.

++ Includes the +++ and ++++ produced by the Kahn and M.L. Tests.

M.L. = Modified Laughlen Reaction.

tested in amounts from 0.01 cc. to 0.1 cc. From the results obtained it would appear that the original ration of 1 : 1 is closely substantiated with the proviso that with the present antigen clearer readings were found when the amount of antigen was slightly less than the amount of serum (0.04 cc. to 0.05 cc.).

There is no doubt that for rapid working these quantities are best measured by "dropping" pipettes. Pipettes of the Dreyer type are easily obtained ready calibrated at 0.05 cc. per drop; this figure refers to physiological saline or serum. To alter one of these pipettes to deliver 0.04 cc. of the antigen is a simple procedure involving only a slight modification of the orifice in a bunsen flame. The calibration is tested by measuring the volume produced by 200 drops of the antigen from this modified pipette and calculating the volume of each drop.

(b) Sensitivity.

As a preliminary test of sensitivity, four antigens were prepared and tested in parallel with the Wassermann and Kahn Tests at the following times after manufacture - 1 hour, 6 hours, 12 hours, 24 hours, 30 hours, 36 hours, 48 hours, 4 days, 10 days, 21 days, 2 months, 3 months. On each occasion of testing, 50 selected sera were used (Table 8). The first point noticed in the table is the remarkably uniform results which were obtained with the four antigens; in each case the

Table 9.

The Effect of Sensitisation with Ten Per Cent Saline

	One Hour ++	±	-	Six Hours ++	±	-	Twelve Hours ++	±	-	Twenty-Four Hours ++	±	-	Thirty Hours ++	±	-
Wassermann	13	5	32	13	5	32	13	5	32	13	5	32	13	5	32
Kahn	13	7	30	13	7	30	13	7	30	13	7	30	13	7	30
M.L.1 ...	6	-	44	9	3	38	11	6	33	13	6	31	13	6	31
M.L.S. ..	10	3	37	12	4	34	13	2	35	13	7	30	13	9	28

	Thirty-Six Hours ++	±	-	Forty-Eight Hours ++	±	-	Four Days ++	±	-	Ten Days ++	±	-
Wassermann	13	5	32	13	5	32	6	1	43	15	3	32
Kahn	13	7	30	13	7	30	6	-	44	17	1	32
M.L.1 ...	13	7	30	13	7	30	6	4	40	17	-	33
M.L.S. ..	13	9	28	16	10	24	9	19	22	21	16	13

M.L.1 = The same M.L.1 Antigen of Table 8.

M.L.S. = A modified Laughlen Antigen sensitised with ten per cent. saline (see text).

sensitivity increased gradually for about 36 hours when the results produced were exactly similar to those of the Kahn Test. It seems quite obvious that this is the point at which full sensitivity develops in the modified antigen. Secondly, the later testings show that this level of sensitivity is maintained for the considerable period of three months. The fact that the level of sensitivity is maintained for so long is of little practical value but it would indicate that the problem of oversensitivity in the Laughlen Reagent has been surmounted: previous work (Page 28) showed that this was due to the control of the pH value.

(c) Sensitisation with Ten Per Cent Saline:

To find if this final sensitisation was necessary, tests were carried out in parallel with the previous experiments on sensitivity. Portions of the modified Laughlen antigens were "sensitised" by adding ten per cent saline in the proportion of 0.15 cc. saline to 1 cc. of antigen as suggested by Laughlen (1938). These antigens were then set aside for 24 hours and tested with the same sera used in Table 8. The results are shown in Table 9 and it is seen that the most notable feature is that full sensitivity was developed in these antigens when they were 24 hours old but a large number of doubtful results appeared after four days and at the end of six days these "doubtfuls" had become so numerous as to make further testing useless. This obviously represents a process

Table 10

(a) The Behaviour of the Antigen After Storage at Different Temperatures.

	Age of Antigen																	
	2 Days			6 Days			14 Days			21 Days			28 Days			36 Days		
	++	±	-	++	±	-	++	±	-	++	±	-	++	±	-	++	±	-
Wassermann	6	-	14	6	-	14	2	1	17	2	-	18	7	-	13	2	3	15
Kahn	6	-	14	6	1	13	2	-	18	2	3	15	7	-	13	3	3	14
M.L.4° ...	6	-	14	6	3	11	2	4	14	2	6	12	7	4	9	5	6	9
M.L.22° ..	6	-	14	6	3	11	2	1	17	2	1	17	7	-	13	5	-	15
M.L.37° ..	6	-	14	6	1	13	2	1	17	2	2	16	7	-	13	4	-	16

Twenty selected sera tested on each occasion.

M.L. = Modified Laughlen (followed by storage temperature).

(b) The Behaviour of the Antigen After Exposure to About 100 Hours Direct Sunlight.

	Exposure to Sunlight					
	Before			After		
	++	±	-	++	±	-
Wassermann	4	2	24	6	-	24
Kahn	5	-	25	6	3	21
M.L.1	5	-	25	6	2	22
M.L.2	5	-	25	6	2	22
M.L.3	5	2	23	6	4	20
M.L.4	5	-	25	6	2	22

Thirty selected sera tested on each occasion.

M.L. = Modified Laughlen.

of sensitisation which is continuous and it is therefore assumed that the degree of sensitivity is not constant over a period of even twelve or twenty-four hours. For this reason, combined with the fact that the results of the previous paragraph indicate that the modified antigen attains a satisfactory level of sensitivity without the addition of strong saline, the final sensitisation with ten per cent. salt solution is considered unnecessary.

(d) Conditions of Storage:

Two main conditions of storage were studied and may be considered under the headings of Temperature and Light. The effects of temperature were studied by storing samples of the modified antigen at 4^{0}, 22^{0} and 37^{0} Centigrade. Tests were made from time to time, the findings of which are shown in Table 10 (a). Little difference was found in antigens stored at the three temperatures but it is seen that when stored at 4^{0} Centigrade for more than 14 days an antigen tends to become more sensitive and to produce a greater number of doubtful results. During a period of fine weather, four antigens were left in such a position that they were exposed to direct sunlight for six to seven hours on 17 consecutive days. These antigens, when tested, showed no deterioration in sensitivity or specificity (Table 10 (b)).

From these two experiments it was concluded that neither variations in temperature nor undue exposure to daylight have

Table 11.

The Results Obtained with 200 Specimens of Plasma Compared with those Obtained with the Corresponding Sera.

No. of Specimens	W.R.	Kahn	M.L. (Sera)	M.L. (Plasma)
163	-	-	-	-
12	++	++++	++++	++(+)
6	++	+++	++++	++
8	++	+++	+++	++
3	++	+++	+++	-
2	+	++	+++	+(+)
3	±	-	-	-
1	-	++	++	-
2	-	-	+	-
200				

any noticeable effect of the modified antigen during storage. One possible practical point which may be considered is that these findings indicate that the antigen may be suitable for use under tropical conditions. For routine purposes in this country storage at room temperature in an ordinary cupboard is adequate.

(e) Bile-Stained Sera:

Lane (1944) has suggested that the Laughlen Test may not be suitable for use with any serum which is bile-stained. During the various tests which were performed with the present modification, the results obtained with these sera were very carefully scrutinised. Over 500 specimens, with varying degrees of staining, were encountered without any indication that they reacted to the antigen in any way different from ordinary sera. It is concluded that bile-staining does not constitute an objection to the examination of such sera by the present modification.

(f) Examination of Plasma:

It has been stated by Laughlen (1938, 1944), that blood plasma gives the same result with the Laughlen Reaction as does serum from the same patient. Two hundred specimens of citrated plasma were examined by the present antigen in parallel with sera from the same patients and it was found (Table 11) that although the results were similar, the plasma from the "positive" patients produced a degree of precipitation

Table 12

Results Obtained with 75 Sera
Before and After Inactivation.

			Modified Laughlen					
			Before Inactivation			After Inactivation		
	W.R.	Kahn	++, +++ or ++++	±	−	++, +++ or ++++	±	−
22	-	-	-	-	22	-	-	22
16	-	++	4	7	5	15	-	1*
8	±	++	-	6	2	8	-	-
4	+	++	1	2	1	4	-	-
18	++	++	6	9	3	17	1*	-
5	++	+++	4	1	-	5	-	-
2	++	+++	2	-	-	2	-	-
(Total) 75								

* Treated Case

very much less marked than that given by the corresponding sera. In three cases of early syphilis, the plasma examinations were negative compared to strongly positive serum reactions supported by positive Wassermann and Kahn Tests. With the present modification, therefore, it was concluded that it was not advisable to use plasma in place of serum and, indeed, there seems little point in so doing even if the findings had been favourable since practically every blood test for syphilis uses serum, and plasma examination would only lead to a cumbersome duplication of specimens. Examination of plasma may prove of some use in the testing of blood donors where it may be convenient to test the supernatant fluid from the citrated blood but even here there is the objection that a positive finding would necessitate obtaining serum for a confirmatory Wassermann Reaction and it seems more convenient to obtain a quantity of coagulated blood in the first instance.

(g) Inactivation of Sera:

That the Laughlen Test does not necessitate the inactiviation of sera was first suggested by Laughlen himself (1938). With the present modification, however, it has been found to be necessary, as is shown in Table 12. Non-inactivated specimens tested with the present antigen produced results which were completely unreliable whereas inactivated sera gave readings directly comparable with the Wassermann and Kahn Tests. Routine inactivation (30 minutes at 56°

Centigrade) is therefore considered necessary.

(h) Testing the Modified Antigen over a Large Series:

The preliminary points relating to sensitivity, storage, inactivation of sera, etc., having been established, the antigen was now subjected to a large-scale trial. In parallel with the Wassermann (Wyler, 1929, 1932) and Kahn (Kahn, 1928) Tests, a total of 16,824 sera were examined by the present modification of the Laughlen Reaction. Of these, 11,496 were from diagnostic (i.e., untreated) cases and 5,328 from cases undergoing antisyphilitic treatment. In the former group, all three tests gave negative results with 10,601 sera and positive results with 374, while 521 sera produced discordant results of one kind or another. These discrepancies are set out in Table 16 and will be discussed later (Page 54). In the "treated" group, 1,460 specimens were positive with the three tests and 2,448 negative. One thousand, four hundred and twenty sera gave discrepancies which are analysed in Table 18 and which are also discussed later (Page 60).

(i) The Effect of Pregnancy:

To assess this the results obtained with 500 sera from pregnant women were compared with those obtained with 500 sera from patients at venereal disease clinics who were undergoing treatment for gonorrhoea. The findings are shown in Table 13. It will be seen that the two groups produced verified positive reactions in 1.6 per cent and 3.4 per cent

Table 13.

(a) Results Obtained with 500 "Pregnancy" Sera

No. of Sera	W.R.	Kahn	M.L.	
8	++	+++ or ++++	+++ or ++++	(Clinically syphilis)
55	-	++	++	
4	-	-	++	
9	-	++	-	
3	±	-	-	
421	-	-	-	
500				

(b) Results Obtained with 500 "Gonnorrhoea" Sera

No. of Sera	W.R.	Kahn	M.L.	
17	++	+++ or ++++	+++ or ++++	(Clinically syphilis)
33	-	++	++	
5	-	-	++	
2	-	++	-	
1	±	-	-	
442	-	-	-	
500				

of cases respectively, these figures being average findings for the City of Glasgow. As described later (Page 57) the + readings were classed as negatives and not examined further, only the ++, +++ and ++++ degrees of precipitation being considered worthy of re-examination by Wassermann and Kahn Tests. Judged by the findings of the Wassermann Reaction, the non-specific rate of the modified Laughlen Test is 11.8 per cent (55 + 4) with the pregnancy sera, and 7.6 per cent (33 + 5) with the specimens from the patients with gonorrhoea. The non-specific Kahn rates were 12.8 per cent (55 + 9) and 7 per cent (33 + 2), respectively. In these two groups, therefore, the non-specific rates were particularly high (vide Page 58) but it is to be noted that in one instance the Kahn figures are slightly higher than those obtained with the modified Laughlen. It is concluded that pregnancy has the effect of producing up to four per cent more non-specific reactions with the modified Laughlen Test than would be found normally, but that this figure is no greater than would be obtained with other precipitation tests, taking the Kahn Test as an average example of the latter.

(j) <u>The Effect of Carcinoma</u>:

Specimens from 129 cases of carcinoma were examined by Wassermann Reaction, Kahn Test and modified Laughlen (Table 14). The surprising feature of this group of sera was the large number of doubtful Wassermann results obtained in cases where

Table 14.

(a) Results Obtained with 129 Sera From Cases of Established Carcinoma.

No. of Sera	W.R.	Kahn	M.L.	
2	++	+++ and ++++	Both ++++	(Clinically syphilis)
26	±	-	-	
8	-	++	++	
2	-	++	-	
91	-	-	-	
129				

(b) Results Obtained with 23 of the 26 W.R. ± Sera Two Weeks After the First Examination.

No. of Sera	W.R.	Kahn	M.L.
19	±	-	-
4	-	-	-
23			

the Kahn and Laughlen Tests were negative. Most of these cases were re-examined, approximately two weeks after the first testing, and it is seen ((Table 14 (b))) that the Wassermann Reaction remained doubtful in 19 of the 23 specimens obtained while both the Kahn and modified Laughlen Tests still gave negative reactions. It is impossible to draw definite conclusions from such a small number of results but the findings suggest that in cases of carcinoma the complement fixation reaction is more likely to produce indefinite or non-specific readings than the precipitation tests.

(k) Malaria:

Sera from only 61 cases of malaria were obtained and little information was gained by their examination, all 61 giving negative results with the Wassermann, Kahn, and modified Laughlen Tests.

(l) Tuberculosis:

Specimens from 127 cases of pulmonary tuberculosis were examined by the modified Laughlen, Wassermann and Kahn Tests. (Table 15). Here again the number of specimens is too small to be of significance: but it is of interest to note that when the cases producing discordant results were examined after an interval of three weeks the only differences found concerned the two sera which were W.R. ± at the first testing - these were both W.R. - at the later examination. Only the one case which gave strongly positive results with all

Table 15.

Results Obtained with 127 Sera
from Cases of Pulmonary Tuberculosis

	W.R.		Kahn		M.L.	
No. of Sera	Initial Test	3 Weeks Later	Initial Test	3 Weeks Later	Initial Test	3 Weeks Later
1	++		++++		++++	
4	-	-	++	++	++	++
2	±	-	-	-	-	-
3	-	-	++	++	-	-
117	-		-		-	
127						

three tests at the first examination produced any clinical signs of syphilis.

Discussion

It is axiomatic in modern serology that the perfect test for syphilis does not exist. Until such a test is developed, therefore, the serologist is forced to use two or more tests, commonly-found examples of which are the various combinations of the Wassermann Reaction and the Kahn Test. As was shown in the introduction to this thesis, one essential feature of such a system in modern practice is the ability to deal with large numbers such as blood donors, and another the power to perform a serum test at short notice as is so often necessary in a city like Glasgow where a rapidly-moving seafaring population frequently presents serological problems. Thus the "screen test" has evolved and it was in this capacity that the modified Laughlen Test was intended for use. To be of real value, the sensitivity level of a screen test must be such that the possibility of a new untreated case escaping detection is reduced to a minimum, even while making allowance for the fact that there is probably no single reaction which can be relied upon to diagnose every infection. It is not one of the functions of a screen test to be used in the control of antisyphilitic treatment but if this is contemplated its sensitivity must in every instance be at least as high as that of any standard test so used. With so much emphasis placed on sensitivity, it is inevitable that non-specific positives are to be expected with a test of this nature but if comparatively few in number they should not cause any great

Table 16.

Analysis of 521 Discrepancies Obtained with Untreated Cases.

Initial Test: No. of Sera	W.R.	Kahn	M.L.	Three Weeks Later: No. of Sera	W.R.	Kahn	M.L.	No. of Sera Not Obtained
260	-	++	-	6	-	++	-	51
				203	-	-	-	
24	±	-	-	22	-	-	-	2
155	-	-	+	64	-	-	-	91
32	-	-	++	3	++	++	+++	2
				17	-	-	-	
				8	-	-	+	
				2	±	++	++	
34	±	+	++	2	++	+++	+++	23
				9	±	+	++	
16	-	+	++	1	++	++	+++	3
				12	-	-	-	
521				349				172

M.L. = Modified Laughlen

inconvenience since the positive "screen" results are confirmed by other reactions such as the Wassermann Reaction, the Kahn Test or both.

The properties of the modified Laughlen Test, therefore, may be discussed in relation to untreated and treated cases and thus the findings of part (h) of the previous section (Page 49) are first reviewed.

Untreated Cases:

This group consisted of 11,496 specimens. Direct agreement between the modified Laughlen (M.L.) Test and Wassermann Reaction was found in 11,235 instances, made up as follows -

Negative with W.R., Kahn and M.L.	10,601
Positive with W.R., Kahn and M.L.	374
Negative with W.R. and M.L. (Table 16) .	260
	11,235

This figure represents 97.7 per cent of the total. (The 34 sera which gave doubtful W.R. readings and ++ readings with the M.L. were not included in this calculation.) Three weeks after the initial testing as many of the discrepant cases as were available were re-examined (Table 16). It was found that agreement now existed in many instances of previous disagreement and that 121 of the available sera now gave similar results with the W.R. and M.L. (The 11 sera giving W.R. ± and ++ M.L. were again ignored.) Though it is hardly permissable to consider these later results as part of the original testing it is of interest to note that if these 121 "late agreements" are added to the "first agreement" total, the correlation between Wassermann and M.L. tests rises to 98.5 per cent. When compared to the Wassermann Reaction, however, the most important property of a screen test is not so much the percentage agreement as the question of whether the screen test is producing negative results with specimens which are Wassermann Reaction positive, or in other words, can the negative results of the screen test be taken as reliable evidence of the absence of infection? In the present series it would appear that the modified Laughlen Test fulfils this requirement. No serum was found to give a positive Wassermann Reaction without also being strongly positive with the modified Laughlen and one feature of the series which is of interest is the group of 24 sera which initially gave doubtful reactions with the Wassermann Reaction and were negative with the modified Laughlen. Three weeks later 22 of this group were negative to both tests indicating an initial non-specific response to the complement-fixation reaction which was not registered by the precipitation test. (Non-specific modified Laughlen Reactions are discussed later.)

Considering early diagnosis, there is no evidence of an earlier specific response to the Wassermann Reaction than to the modified Laughlen Test, while there are some grounds for believing that the reverse may be true. In 48 (32 + 16) instances where the initial findings were W.R. - and M.L. ++, subsequent examination showed that at least four of these specimens were from cases of syphilis, indicating that in these, admittedly few, cases the modified Laughlen gave a definitely earlier response than did the Wassermann. It is difficult to assess the findings of the group of 34 sera whose initial reactions were W.R. ± and M.L. ++ as only 11 of these specimens were available for retesting; two of these sera proved to be from definite cases of syphilis while the remaining nine gave the same reponse as before. The only comment which may be made on this group is that both tests gave early indication of infection in at least two instances. Judged by these findings, therefore, the modified Laughlen Test does not lack the ability to give a specific reaction at least as early as the Wassermann Reaction.

When the Kahn Test figures are examined, the outstanding feature is the large number of specimens (260) which gave Kahn ++ but which were negative to both Wassermann Reaction and modified Laughlen. The later examinations showed that over 200 of these sera had given non-specific Kahn positive reactions originally. Fifty-one sera from this group were not re-examined but there seems no doubt that at least a large majority of the original tests may be classed as non-specific Kahn positives. With all these sera the modified Laughlen had produced uniformly negative results which were supported by negative Wassermann Reactions. As with the Wassermann, there is no evidence that the Kahn Test gave a specific positive response earlier than the modified Laughlen, while in at least three instances the modified Laughlen reacted earlier than the Kahn Test. No serum was found to give a specific positive result with the Kahn without also being positive with the modified Laughlen. The percentage agreement with the two tests was approximately the same as with the Wassermann Reaction and modified Laughlen - 97 per cent on the initial testing and 98 per cent if the "three weeks later" results are taken into account.

At this point it is convenient to discuss the various grades of precipitation produced by the modified Laughlen Test. As is seen in Table 16 the "doubtful" grade was produced with 155 sera which on later testing gave negative readings with 64, the remaining 91 specimens being unobtainable. This suggests that the + reading (in untreated cases) may be regarded as negative and in fact this has been substantiated on many subsequent occasions. Several hundred of these + readings have been encountered since the results of this series were analysed and in no instance was this result associated with any indication of positivity in either the Wassermann Reaction or Kahn Test initially or at re-examination. With this version of the Laughlen Test therefore, all grades of precipitation which require the x6 lens for their detection

Table 17.

Analysis of the 374 Positive Results

No. of Sera	W.R.	Kahn	M.L.
262	++	++++	++++
37	++	+++	++++
56	++	+++	+++
1	++	++	++
18	++	++	+++
374			

are classed as negative in the untreated case.

Table 17 is a partial analysis of the 374 positive results in order to show what grades of M.L. precipitation were produced with the various sera. Two hundred and ninety-nine of these specimens gave a reading ++++ while a further 74 gave +++, a combined figure of more than 99 per cent of the total positives. The total of ++ reactions found in the series was 83 (82 in Table 16 and one in Table 17). Of all the ++ readings therefore, one in 83, or 1.2 per cent, were associated with positive reactions with the other tests. On the basis of the present series then, 99 per cent of cases of untreated syphilis will give either a ++++ or a +++ reading with the modified Laughlen while roughly one in every 100 ++ reactions is also associated with active infection.

The non-specific rate of the modified Laughlen, like every other non-specific rate, is difficult to assess and its accurate calculation would involve the follow-up of many thousands of cases. Some tentative information, however, may be gained from the figures of the present series. The first point to be noted is that every ++++ or +++ result is associated with strongly positive readings with the other tests, i.e., a ++++ or +++ reading with the modified Laughlen is completely specific. There is a sharp division between these readings and the ++ grade in respect of specificity. As has been shown in the last paragraph, only about one per cent of the ++ readings were specific according to the initial testing. Thus it is seen that when the specificity of the modified Laughlen is under consideration each grade of positivity must be considered separately in the same way as a + Wassermann Reaction is not accorded the same unquestioned specificity usually associated with ++ result in the same test. In other words, the phrase "a positive M.L." is meaningless since it may be construed as a ++++ result which would definitely indicate syphilis, or as a ++ result which, <u>per se</u>, would indicate nothing; the <u>degree</u> of positivity is the important point and must always be stated in order to gain a true impression of the reaction given by any particular serum.

From these findings with a large series of "diagnostic" sera the modified Laughlen Test has been proved to be satisfactory. Sensitivity was 100 per cent and it may be claimed with confidence that no case of syphilis was missed by the modified Laughlen when it would have been diagnosed by the other reactions. The important points concerning specificity have been stressed above, and while complete specificity is neither sought nor expected in a sensitive screen test, it is of value to know that every +++ or ++++ modified Laughlen reaction indicates syphilis and that these grades of positivity constitute more than 80 per cent of all recorded positives (373 out of a total of 456 specimens giving ++, +++ or ++++).

Table 18.

One Thousand, Four Hundred and Twenty Discrepant Results With Sera from Treated Cases.

	W.R.	Kahn	M.L.
765	-	-	++
582	-	++	++
37	-	++	-
6	+	++	+
13	±	++	-
17	±	-	++
1,420			

M.L. = Modified Laughlen.

<u>Treated Cases</u>:

This group consisted of 5,328 sera of which 3,945 showed agreement with Wassermann Reaction and modified Laughlen -

Negative with W.R., Kahn and M.L.	2,448
Positive with W.R., Kahn and M.L.	1,460
Negative with W.R. and M.L. (Table 18) ..	37
	3,945

This figure represents 74 per cent of the total, the "doubtful" results being omitted from the calculation. This rather low rate of agreement is no doubt due, at least in part, to the many instances (765 - Table 18) in which the modified Laughlen remained positive when both the Wassermann Reaction and Kahn had become negative.

With the Kahn Test the modified Laughlen agreed in 4,490 instances -

Negative with W.R., Kahn and M.L.	2,448
Positive with W.R., Kahn and M.L.	1,460
Positive with Kahn and M.L. (Table 18) ..	582
	4,490

representing 84 per cent of the total.

The interesting feature of these "treated" results is the irregular behaviour of the modified Laughlen when compared to the reactions of the other two tests and it must be remembered that these tests, the Wassermann and Kahn, are the accepted standards of antisyphilitic treatment control. In 13 cases where the Wassermann Reaction was still showing a doubtful result, i.e., "residual positivity", the modified Laughlen was negative, while 50 sera (37 + 13) were Kahn ++ and modified Laughlen negative. This latter finding would suggest that about one per cent of treated cases give no reaction to the modified Laughlen while still showing signs of the infection by the results of the Kahn Test, a widely-used treatment control. In contrast to this, 765 cases whose treatment would have been deemed satisfactory by the negative findings of the Wassermann and Kahn, remained positive (++) with the modified Laughlen.

From the results obtained it seems clear that the modified Laughlen is of little or no assistance when testing treated cases as there is a marked lack of agreement with the findings of the Wassermann and Kahn Tests.

The Other Properties of the Antigen:

Most of these have been partly discussed under the particular headings of the previous section.

The fact that the sensitivity level of the modified antigen remains so constant for such a long period has one very useful application since it enables the serologist to perform a rapid test for syphilis within a few minutes of receiving the inactivated serum. No preparation of antigen is necessary; the modified Laughlen antigen is simply shaken to restore its colloidal appearance and is ready to be used provided it is at least 36 hours old.

Storage of the antigen presents no problems and variations of warmth and light do not appear to affect the antigen adversely.

Contrary to what has been stated by Laughlen (1938 and 1944), inactivation of sera is necessary, at least with this version of the test. This is in direct agreement with the findings of Nelson (1941) who based his conclusions on the results of a large series and decided that inactivation was essential for sera to be tested with the Laughlen Reaction.

In view of the fact that this investigation was originally undertaken to find a rapid test for the examination of specimens from ante-natal clinics, the results obtained with the "pregnancy" sera were especially interesting. As was expected the non-specific rate was relatively high but was no greater than that of the Kahn Test. In this connection it is to be noted once more (Table 13) that all +++ and ++++ modified Laughlen results were completely specific.

The number of specimens which were available from cases of carcinoma, malaria and tuberculosis was too small to be of any statistical value but there was no evidence to suggest that the modified Laughlen is not suitable for use with sera from such patients.

Conclusions

The present modification of the Laughlen Test for syphilis has been found to be completely reliable as a screen test for untreated cases. The antigen is simple to prepare and of constant sensitivity; the apparatus is easily improvised and the test is simple and rapid in performance. Since the antigen may be stored almost anywhere it is suggested that this version of the Laughlen Reaction would be of value in a small laboratory where a diagnostic Wassermann Reaction would be impracticable. The speed and simplicity of the test, together with its reliability, make it an excellent method of conducting research, as will be demonstrated in Section Two.

The irregular results obtained with treated cases would suggest that this modification of the Laughlen Test is unsuitable for testing the sera from patients undergoing antisyphilitic treatment; in the present instance this is of minor importance since the test was intended for use as a rapid method of diagnosis in the untreated case.

Addendum to Section One

As an additional assessment of the value of the modified Laughlen Test just described, the results obtained by an independent worker are of interest. Dr. T. Gow Brown, Bacteriologist to the County of Lanark, who has had extensive experience of several versions of the Laughlen Test, conducted trials with the present modification; the reagents used in these trials were made personally by him according to the formula described on page 35. The total number of specimens examined was 5,453; of these 5,080 were "diagnostic" sera, and 373 from patients undergoing antisyphilitic treatment.

In the diagnostic group, 5,022 sera showed complete agreement between Wassermann Reaction and the modified Laughlen Test (M.L.), representing 98.8 per cent of the total. Of the 58 discrepancies, seven were W.R. negative and M.L. ++ and were classed as non-specific M.L. positives; the remaining 51 sera gave W.R. doubtful and M.L. negative and as these cases showed no signs of the disease and were serologically negative on later examination it was concluded that the slight degree of complement-fixation obtained was purely non-specific. No case of syphilis was diagnosed by the Wassermann Reaction which did not at the same time produce a strongly positive result with the modified Laughlen. Agreement with the Kahn Test was found in 5,077 instances (99.9 per cent).

In the treated group, agreement with the Wassermann and Kahn Test was found in 324 (86.8 per cent) and 347 (93.3 per cent) cases, respectively. Eighteen known cases of syphilis undergoing treatment were positive with the Wassermann Reaction and negative with the modified Laughlen, while 31 were W.R. negative and M.L. positive - of these, 15 were also Kahn negative.

While it is not intended to analyse Dr. Gow Brown's results in full, it is seen in this brief summary that the findings are very similar to those described in Section One (pp. 54 and 61). Reliability with the untreated cases is apparent, while it is demonstrated once more that with treated cases the modified Laughlen sometimes gives negative results while the Wassermann and Kahn reactions are still positive, yet in many instances it remains positive when the results of these two tests indicate that the case has been adequately treated. These findings support the view that the modified Laughlen Test is suitable for use with sera from "diagnostic" cases but that false negatives may occur with sera from cases undergoing antisyphilitic treatment.

Dr. Gow Brown further stated that he found the modified reagent simple to use and that its performance was constant over a period of "at least ten weeks".

I have to thank Dr. Gow Brown for permission to make use of his results.

SECTION TWO

INVESTIGATIONS ON SYPHILIS ANTIGEN

(a) The Value of Soya Bean Extracts.

(b) The Significance of the Diaminomonophosphatide Sphingomyelin.

(a) The Value of Soya Bean Extracts.

Although animal tissues, such as ox heart and human heart or liver, are the usual sources from which syphilis antigens are obtained, there is no theoretical reason why these antigens should not be extracted from plants, since the phosphatides, which presumably form the essential elements of these antigens (vide Page 94), are present in all living matter, both animal and vegetable. References to such work are surprisingly few in number. In 1912, extracts of potato were examined by Heinlein who reported that their antigenic activity in serological tests for syphilis was quite marked but was soon lost; presumably because of deterioration during storage. Tribondeau (1913) tested extracts made from peas, beans and corn and found that extracts of pea produced the best antigens. A wide variety of vegetable extracts was investigated by Weiss (1923), who reported that good antigens could be made from olives and coco-nuts, fair antigens from the seeds of ricinus, flax, hemp, radish, sunflower, poppy and rape; and from peas and mustard, but he found no antigenic activity in corn, walnut, beans, barley, lentils or in the seeds of water-melon and squash. Walker (1917) mentioned a satisfactory antigen having been made from peas.

All these extracts were used as antigens in the Wassermann Reaction and so far as can be ascertained no attempt has ever been made to use a vegetable extract as an antigen for a precipitation test. Judging from the reports of the writers quoted this is not surprising since the impression gained is that while certain plants contain "antigenic" substances which can be extracted, the antigens obtained are not altogether satisfactory and from the continued popularity of animal tissue extracts it is evident that the latter possess the advantages of being conveniently obtainable, constant in performance, and of high antigenic activity. Nevertheless, the fact remains that syphilis antigens have been extracted from vegetable matter, and therefore there may exist some plant with impressive antigenic potentialities.

To be of real value, a plant used as a source of antigen must fulfil certain conditions apart from the ability to provide good antigen. It must be capable of being cultivated on a relatively large scale under conditions which are standardised as far as possible; it must be easily available at low cost; and the extraction of antigen from the plant must be a comparatively simple process. There is one plant which largely fulfils these conditions - the soya bean - with the obvious proviso that its capacity to provide antigen is not known. The soya bean is widely cultivated in many parts of the world and it is easily obtainable though at present subjected to a degree of Governmental control by reason of its oil content: its cost is reasonable and the phosphatide content is known to be relatively high. These features will be more fully considered later but it is obvious that the soya bean was promising material to investigate. Since many methods of extraction might have to be tried and many antigen combinations prepared, it was necessary to select a simple, quick and convenient method of examination. The modified Laughlen Test (Section One) appeared eminently suitable for this purpose.

<u>A Note on the History of the Soya Bean</u>:

As a food, the soya bean plant (Soja hispida) has been cultivated for centuries in Eastern Asia. The first accounts of its use are to be found in ancient Chinese writings set down more than 2,000 years before the Christian era (Markley and Goss, 1944) and it was probably from the Chinese that the rest of the world gradually came to appreciate the benefits of this versatile plant. Its uses as a food are almost too numerous to describe, and it was as a food that it was known until comparatively modern times when large-scale investigations revealed that apart from its nutritional values, the soya bean contained many substances which could be extracted and used in the manufacture of an almost endless number of by-products, ranging from cellulose and preservatives to fertilisers and high explosives. Soya bean oil is now an important source of fats and is used in many instances to make up for the present-day shortages of animal fat.

The cultivation of the bean outside its native Asia proved difficult at first but in the period 1920-30 it was grown on a large scale in the United States of America, which now produces a good annual crop. Cultivation of the plant is at present being undertaken in many countries now that its multiple uses are recognised (Markley and Goss, 1944).

<u>The Composition of the Soya Bean</u>:

Most authorities agree that the composition of the soya bean varies with its place of cultivation. (Bowdidge, 1935). The main constituents, however, do not vary widely. One analysis, quoted by "The Lancet" in 1940, gives the following figures -

Protein	-	40 per cent.
Fat	-	20 per cent.
Carbohydrate	-	20 per cent.

Later calculations (Markley and Goss, 1944) state the composition as -

Protein	-	40 per cent.	
Fat	-	18 per cent.	
Pentoses	-	4.4 per cent.)	
Sugar	-	7 per cent.)	17 per cent.
Starch	-	5.6 per cent.)	
Moisture	-	8 per cent.	
Fibre	-	3.5 per cent.	
Ash	-	4.6 per cent.	

Total phosphorus content - 0.59 per cent.
Phosphorus per gramme of bean - 5.6 milligrammes.
Phosphatides in soya-bean oil - 1.5 to 2.5 per cent.

In 1927 the phosphatide content had been given as about 1.6 per cent (Maclean, 1927) and as this agrees closely with the lower figure given in 1944, it may be assumed that the average phosphatide level is about 2 per cent, a high value when compared to most other plants, e.g., 0.8 per cent in the

Figure 4.

Soya Beans.

case of barley and linseed (Maclean, 1927). The fact that the bean has such a relatively high phosphatide content suggests that it might be possible to extract syphilis antigen of fair quality.

Commercially, the bean is available in two forms (a) as the whole bean (Figure 4), and (b) as "soya flour". The flour is used for a variety of cooking purposes and is much more easily obtainable than the bean. It is produced when soya oil is extracted from the whole bean and thus represents the crushed bean from which most of the oil has been taken (Elsdon, 1926). Soya flour is yellow in colour and is slightly oily to the touch. It has a powdery appearance but the particles are quite adherent to each other (no doubt due to traces of oil), and there is thus no tendency to dustiness as with the flour of wheat. The texture is similar to that of powdered linseed.

The dried bean has an average diameter of about a quarter-of-an-inch: it is roughly spherical and is very hard. Its colour is pale brown or yellow and it has a tough outer skin.

The Methods of Extraction

Extracts were made from the two forms in which the plant is readily available - (a) the whole bean, ground to powder by hand, and (b) the commercial "flour". Several different methods of extraction were used to find the most suitable or most promising procedure. Naturally, these methods were largely empirical but they were based on the established principles of syphilis antigen extraction and on the known solubilities of the phosphatides. In all, fourteen extracts were produced, seven from soya flour in parallel with seven from the whole bean. The flour extracts are described first.

Extract No. 1:

It seemed reasonable to suppose that it might be possible to extract antigen from soya flour in much the same way as Kahn antigen is extracted from dried beef heart and the first extraction was made in this way.

Twenty-five grammes of soya flour were substituted for the dried heart and treated as described on Page 20. (The four ether extractions were kept for further examination. They were pooled, placed in a 500 cc. flask in an incubator at 37° Centigrade and allowed to evaporate.) The final alcoholic extract, named S1, was straw-coloured and had a faintly aromatic odour; 64 cc. were obtained. The usual amount of cholesterol was added to 20 cc. of this extract, i.e., 6 mgm. per cc., a total of 0.12 gm., and the first attempt to produce a "Laughlen" reagent from this extract was made.

Table 19.

The Reactivity of S1L Over a Period of Three Weeks

			Age of S1L												
			Hours							Days					
Serum	W.R.	Kahn	6	12	18	24	30	36	48	3	5	8	12	21
1	-	-	-	-	-	-	-	-	tr.	++	++	All readings of a granular character and unreadable		
2	-	-	-	-	-	-	-	-	tr.	+	++			
3	-	-	-	-	-	-	-	-	tr.	++	++			
4	-	-	-	-	-	-	tr.	tr.	tr.	++	++			
5	-	-	-	-	-	-	-	-	tr.	+	++			
6	+	++	-	tr.	+	++	++	++	++	++	++			
7	++	++	tr.	tr.	+	+	++	++	++	++	++			
8	++	++	-	tr.	++	++	++	++	++	++	++			
9	++	+++	tr.	+	++	++	++	++	++	++	++			
10	++	+++	tr.	++	++	++	++	++	++	++	++			

tr. = trace of precipitation.

The reagent was made in exactly the same way as has been described in Section One, except that the soya extract took the place of the Kahn antigen. A smooth emulsion was obtained, indicating that the extract at least contained lipoids of some description since it has been found that a good emulsion is never obtained with a Laughlen antigen unless a fair amount of lipoids are present in the original alcoholic extract.

The antigen, designated S1L, was laid aside for an empirical period of six hours, following which tests were made to see if it would react with syphilitic sera. At this point it was noticed that no sedimentation had occurred; later, when the antigen was some 18 hours old, there was definite sedimentation though of a more flocculant type than that found when Kahn antigen is used. The tests were carried out at six-hourly intervals, using ten sera, five of which gave negative reactions to the Wassermann and Kahn Test and five giving varying degrees of positivity with these two reactions (Table 19).

The results of this first testing showed clearly several interesting features. First, there was no doubt that the modified antigen reacted with syphilitic sera and thus the extract of soya flour must have contained substances constituting syphilitic "antigen". (A control reagent prepared from alcohol containing cholesterol produced a poor emulsion giving negative results.) Secondly, the modified reagent did not become fully sensitive until 24 to 36 hours old; and thirdly, over-sensitiveness developed when it was about 48 hours old which made the reagent useless, approximately three days from the time of manufacture.

This initial experiment was of great interest. The results could be compared with those of Heinlein who had found (Page 66) that potatoes contained an antigenic substance but that the activity of extracts was soon lost. In the present instance the problem appears to be not loss of activity but the development of an over-sensitiveness which is obviously not controlled by the use of buffer-saline and Pyrex glass as were the Laughlen reagents made from Kahn antigen, and thus it may be assumed that the alcoholic soya extract contained some substance capable of reacting with syphilitic sera but when combined in an antigen such as has been described (S1L) for a period of 72 hours, it caused positive reactions with all sera, syphilitic or not. This implies some sensitising process which, instead of rising to a fixed level, continues to develop until the antigen is producing valueless results.

The hypersensitivity developed in the prepared antigen and is therefore no indication of any similar process taking place in the cholesterolised soya extract itself. As a test of this, a second "Laughlen" reagent (S1L2) was made when the

Figure 5.

Estimation of the "Kahn" Titre of S1.

Stage 1:

Varying amounts of saline mixed with a constant quantity (0.5 cc.) of S1 in small test tubes.

S1 (cc.)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaCl (cc.)	0.6	0.65	0.7	0.75	0.8	0.85	0.9	0.95	0.1	1.05	1.1	1.15

These mixtures were left for 30 minutes then 0.025 cc. of each emulsion placed in a small test tube and 0.05 saline added:-

Stage 2:

The 12 tubes were next shaken for three minutes on a standard Kahn "shaker" and to each was added 0.5 cc. of saline.

Stage 3:

The tubes were then examined by concave mirror for the presence of visible aggregates. These were found to be present in the tubes containing the smaller amounts of saline (Stage 1) and absent in those containing the greater amounts. The line of demarcation occurred sharply, aggregates being visible in the tube containing the emulsion made with 1.05 cc. saline and not seen (i.e., "dispersed") in the tube containing the emulsion made with 1.1 cc. saline. The titre of S1 is therefore 1.1 cc. saline to 0.5 cc. S1.

(The saline used in all stages was 0.85 per cent NaCl.)

extract was seven days old and the reactivity tested with ten sera as was S1L. Almost exactly similar results were obtained, i.e., hyper-sensitivity developed when the antigen S1L2 was some 72 hours old, indicating that the changes in sensitivity occurred when the extract was combined as a sensitised antigen.

As this first extract, S1, evidently contained antigenic substances and as it had been produced from soya flour in the same way as Kahn antigen is made from dried heart muscle, an attempt was now made to discover if it could be used in place of Kahn antigen in the Kahn Test proper. This involved first the estimation of the titre as described on Page 21, to find the least amount of saline which when added to the extract to make the antigenic emulsion would produce complete dispersion of the antigenic particles in the presence of normal sera. The method is demonstrated in Figure 5 and it is seen that the titre reached the extremely high figure of 1 : 2.2. With Kahn antigen made from dried heart, the figure is normally about 1 : 1.1. It seemed unlikely that an emulsion produced by adding 2.2 cc. of saline to 1 cc. of extract would give reliable results and in fact this was borne out by tests. A "Kahn" antigen emulsion was made from the soya extract at the titre stated and a standard "Kahn" test carried out with 20 sera of known reactivity. The results are shown in Table 20 where it will be seen that while

Table 20.

The Results Obtained Using S1 in Place of the Antigen in the Kahn Test. Twenty Selected Sera Employed.

Serum	W.R.	Kahn Test Proper	"Kahn" Test Using S1
1	-	-	+(+)
2	-	-	-
3	-	-	-
4	-	-	+
5	-	-	±
6	-	-	+
7	-	-	+
8	-	-	-
9	±	++	+
10	+	++	++
11	++	++	++
12	++	+++	++
13	++	+++	+(+)
14	++	+++	++
15	++	+++	+++
16	++	++++	+++
17	++	++++	++++
18	++	++++	++++
19	++	++++	++
20	++	++++	+++

strongly positive reactions were obtained with "positive" sera, varying degrees of precipitation were found with "negative" specimens. Similar results were obtained when the titre was tried at 1 : 1.6 and 1 : 2.8. From this experiment two conclusions were drawn (a) there is further evidence that the soya extract contained syphilis antigen, judging by the strongly positive reactions obtained with sera from known syphilitics, and (b) there is present in the soya extract some "sensitising factor" which gives rise to false positive reactions and which may be considered similar to, if not identical with, the factor causing the development of over-sensitiveness in the "Laughlen" reagents S1L and S1L2.

The ether-soluble fraction obtained in the production of S1 (Page 71) was now examined to discover if it contained any antigenic substances. The ether had rapidly evaporated, leaving a syrupy liquid which amounted to 23 cc.; 100 cc. of ethyl alcohol was added to this syrup, the mixture shaken and left standing at room temperature for 30 minutes. The alcohol was then filtered off and cholesterolised, this extract being designated E1. A modified Laughlen reagent was prepared and tested with 20 selected sera, the results of which are shown in Table 21. It can be seen that this reagent was almost completely insensitive and only produced a few doubtful readings among those sera which were strongly positive with the Wassermann and Kahn Tests. Attempts to

Table 21.

The Results Obtained with a Reagent Made from E1.

Serum	W.R.	Kahn	E1L
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	+	++	-
12	+	++	-
13	+	++	-
14	++	++	-
15	++	++	+
16	++	++	-
17	++	+++	+
18	++	+++	+
19	++	+++	-
20	++	++++	+

increase the sensitivity by altering the cholesterol and NaCl content, etc., as described on Page 80, were unsuccessful and it was concluded that these ether extracts contained only a trace, if any, of antigenic principle.

Extract No. 2:

It has been established (Maclean, 1927), that the phosphatides are more soluble in hot alcohol than in cold and this second extraction was made with this fact in mind. The method was similar to that used for Extract No. 1, the only difference being that the alcohol extraction was made at boiling point (80° Centigrade); by this method it was thought that a greater yield of antigenic substance might could be expected.

After the ether extractions, the material was dried as before and placed in a 250 cc. flask which had been weighed. The exact weight of the tissue was thus obtained (21.5 gms.) and to the flask was added 107.5 cc. of 95 per cent ethyl alcohol (5 cc. for every gramme of material). The flask was now placed in a water-bath at 80° Centigrade and in a few minutes the mixture was boiling; this was maintained for one hour. The alcohol was filtered off while hot, made up to its original volume and placed in an ice-chest at 4° Centigrade overnight. A white deposit of phosphates had settled out by the following morning - these were removed by filtering through Whatman's No. 1 paper. The filtrate, pale yellow in colour, was cholesterolised in the usual concentration and labelled "S2".

As before, a "Laughlen" reagent (S2L) was made from this soya extract and tested out over a period of three weeks in the same way as with S1L. The results were similar in that the reagent became oversensitive after about 48 hours but there was one outstanding difference; this concerned the degree of precipitation obtained with the positive sera. With S1L, ++ was the top limit of precipitation, even with sera which gave a ++++ with the Kahn Test, but with S2L the precipitation was much more marked and all positive specimens reacted +++ or ++++ within 18 hours of the reagent having been made, while the negatives were particularly clear and "colloidal". These results appeared to indicate that S2 was a better extract antigenically than S1 and it was therefore tried out on a larger scale. A fresh reagent was made, designated S2L2 and left for 18 hours before use. The test sera consisted of 143 specimens which were about to be tested routinely by Wassermann and Kahn Tests. The results obtained are shown in Table 22. In view of previous experience, the sera were considered in two groups - "Diagnostic" and "Treated" - consisting of

Table 22.

Results Obtained with Wassermann, Kahn and S2L2 Over 143 Routine Specimens.

No.	W.R.	S2L2	Kahn
Diagnostic Sera			
45	-	-	-
4	++	+++	+++
3	-	+	-
52			
Treated Sera			
54	-	-	-
14	-	++	++
16	++	++	++
2	±	++	++
2	++	-	++
1	+	++	++
1	±	-	-
1	+	-	++
91			

Total - 143.

52 and 91 specimens, respectively. In the diagnostic group it is seen that there was complete agreement with the other tests except the three sera giving a + result with S2L2 and negative results with Wassermann and Kahn; in accordance with the previous findings, these three + results may be ignored for practical purposes. With the treated cases the findings were quite interesting. While there was a general agreement of results with most of the specimens, two sera which gave Wassermann ++ and Kahn ++ were negative to S2L2, while one specimen giving Wassermann +, Kahn ++ was also negative to S2L2. Although the series is small, these results suggest that this reagent suffers from the same defect as the modified Laughlen reagent (Section One) in that it fails to react with a certain percentage of "treated" specimens which give positive results with other tests. With diagnostic sera the results seem to be completely reliable. These findings in themselves might almost be taken as evidence that the antigenic substance present in the soya extracts was of a similar nature to that found in beef heart antigens of the Kahn type.

As was done with S1, attempts were now made to use S2 as a "Kahn" antigen. The titre was estimated as before and was found to be 1 : 2.2. Tests similar to those in Table 20 were carried out with somewhat better results than were obtained with S1. The degrees of precipitation were more clearly defined with the positive sera and there were fewer doubtful readings with the negatives but in general the results were poor.

Extract No. 3:

It is known (Page 101) that the phosphatides are practically insoluble in acetone and thus it seemed reasonable to expect a "purer" yield in alcoholic extracts made from tissues which had been previously treated with acetone to remove fatty substances. This "purification" of antigenic extracts has been attempted on many occasions since it was first tried by Noguchi (1909).

To 50 gms. of soya flour in a 500 cc. flask was added 200 cc. of acetone. The flask was placed in the ice-chest at 4° Centigrade (the phosphatides being slightly more soluble in warm acetone than in cold) and left for three days: the contents were well mixed by shaking vigorously for five minutes each day. Separation of the acetone was carried out by double filtration when a deep yellow liquid was obtained: the residue was completely dried by being spread out on a glass plate and left in a 37° Centigrade incubator overnight.

Again using empirical quantities, the acetone-extracted flour was placed in a 250 cc. flask containing 150 cc. ethyl alcohol which was heated to 80° Centigrade in a water-bath for one hour as with the second extraction. After the alcohol had been separated and the phosphates removed, the pale yellow extract was cholesterolised and designated S3.

Experiments with this latest extract proved disappointing. When a "Laughlen" reagent was made it was found to be watery in appearance and it lacked the smooth colloidal texture of other reagents; as might be expected this reagent was over-sensitive and produced a granular precipitation with all sera, positive or negative. Using S3 as a "Kahn" antigen was equally of no value, precipitates appearing with all negative sera.

In an attempt to discover the reason for the poor results obtained with this third extract, the methods of preparation were varied -

(a) Three separate acetone extractions were made, each of 24 hours' duration, followed by hot alcohol;

(b) As in (a) above but the alcohol extraction was made at room temperature for three days;

(c) The amount of cholesterol used was varied;

(d) The amount of diluting saline used to make the modified reagents was varied - 5 cc., 8 cc., 12 cc., 15 cc., in place of the 10 cc. normally employed;

(e) The NaCl content of the diluting saline was varied - 0.5 per cent, 0.7 per cent, 1.5 per cent, 5 per cent, in place of the standard 0.9 per cent.

None of these methods succeeded in producing an antigen giving good results. All the reagents suffered from the same fault of being oversensitive. It seemed clear that preliminary extraction with acetone did not "purify" the soya flour satisfactorily or perhaps removed the antigenic substance; this seemed unlikely since the phosphatides are almost insoluble in acetone. Nevertheless, the acetone filtrates, all of which had been laid aside in an incubator at 37° Centigrade, were now examined. It was found that the acetone had evaporated leaving thick, green-brown syrups, slightly opalescent and with a peculiar "fatty" odour - the several syrups were practically identical. To each of these acetone-soluble extracts (four in all) was added 150 cc. of ethyl alcohol and the mixtures were shaken vigorously. Cloudy yellow precipitates formed and after standing at room temperature for 30 minutes, the alcohol was separated off by filtration and was found to be pale yellow in colour. Thus the acetone-soluble fractions could be split into alcohol-soluble and alcohol-insoluble portions.

The use of these alcohol filtrates as antigens produced negative results. The "Laughlen" reagents were colloidal in appearance but no precipitates were formed with positive sera; similar results were obtained when the cholesterol, strength of saline, and amount of saline were varied as described above.

From these findings it was concluded that no antigenic substance is removed from soya flour by a preliminary acetone extraction, because a subsequent alcohol extraction of the acetone-soluble fraction does not produce an extract possessing antigenic substances in appreciable amounts.

Extract No. 4:

Having examined alcoholic extracts which were made after preliminary treatment of the flour with ether and acetone, a trial was now made using alcohol only, i.e., ommitting any previous extraction with acetoneor ether. Fifty gms. of the flour were placed in a 500 cc. flask containing 200 cc. of ethyl alcohol and extraction was allowed to proceed for seven days at room temperature. The alcohol was separated and the phosphates removed as before and the usual concentration of cholesterol added. Once again negative results were obtained. The reagents produced from this extract (S4) had no colloidal quality whatsoever and granular precipitates were obtained with both positive and negative sera. Extraction with hot alcohol gave no better results nor did variation of cholesterol content

NaCl, etc. (Page 80). Direct alcohol extraction therefore was considered unsuccessful in removing antigenic substances from the soya flour. Attempts to use this extract as a Wassermann antigen are referred to later.

Extracts Nos. 5, 6 and 7:

In many of the investigations on the chemistry of syphilis antigen, petroleum ether has been used in the extraction of the active principle which, in common with all the phosphatides, is soluble in this organic solvent. It was now used in the extraction of soya flour; three methods were employed -

(a) Direct Extraction with Petroleum Ether - 50 gm. of the flour were added to 200 cc. of petroleum ether in a 500 cc. flask and extraction allowed to proceed for three days at room temperature. By this time the petroleum ether had evaporated to about half its original volume; it was separated by filtration and evaporated as far as possible by keeping at 37° Centigrade for four days until no odour of petroleum ether could be detected and it was found that the residue consisted of a thick syrup similar to that obtained from the acetone fractions (Page 81).

(b) Treatment with Ether Followed by Petroleum Ether Extraction - 50 gm. of the flour were extracted with ether as described for Kahn antigen (Page 20). When completely dry following the last extraction, the flour was placed in a 500 cc. flask containing 200 cc. of petroleum ether. Thereafter the extraction was completed as in (a).

(c) Treatment with Acetone Followed by Extraction with Petroleum Ether - 50 gm. of the flour was extracted in the cold with 200 cc. of acetone exactly as described for S3 (Page 79) and the subsequent petroleum ether extraction carried out as described in (a) above.

Three "syrups", of each about 27 cc., were thus obtained. They were placed separately in 250 cc. flasks each containing 100 cc. of ethyl alcohol, tightly stoppered, shaken and left at room temperature for two hours. At the end of this time the mixtures were found to consist of clear yellow liquids and whitish flocculant deposits. The supernatant fluids were filtered off, cholesterolised and designated S5, S6 and S7, corresponding to the methods of extraction (a), (b) and (c), respectively.

The examination of these three extracts was carried out as before. Reagents were made, allowed to stand for 18 hours and tested with a number of sera of known reactivity. The results are shown in Table 23. As with previous reagents, the letter "L" after the extract number indicates the "Laughlen"

Table 23.

Results Obtained with S5L, S6L, S7L, Wassermann and Kahn Test with 25 Selected Sera from Untreated Cases.

Serum No.	W.R.	Kahn	S5L	S6L	S7L
1	-	-	-	-	+
2	-	-	-	-	++
3	-	-	-	+	+
4	-	-	-	-	+
5	-	-	-	-	-
6	-	-	-	-	+
7	-	-	-	-	-
8	-	++	-	++	+
9	-	++	-	+	++
10	-	++	+	+	++
11	-	++	+	++	++
12	-	++	++	++	++
13	±	++	+	++	-
14	±	++	+	++	-
15	±	++	+	+	++
16	±	++	+	++	+
17	+	++	+	++	-
18	+	+++	++	++	++
19	++	++	+	+	-
20	++	+++	++	+++	++
21	++	+++	+	++	++
22	++	+++	++	++	++
23	++	++++	++	+++	+
24	++	++++	++	++	++
25	++	++++	++	+++	++

made from that particular extract.

From Table 23 it is obvious that S6L produced the best results with this small series and seemed to occupy an intermediate position between the other two reagents. For the most part S5L was not sensitive enough, while S7L was oversensitive but produced negative results in several instances where the Wassermann and Kahn were positive (Sera No. 13, 14, 17 and 19). It is also to be noted that S6L was the only one of the three to give readings of the +++ grade. When these three reagents were tested three days after manufacture they were all found to be oversensitive and gave positive results of a granular type with all sera.

As was done with S2, S6 was next tested out on a larger scale since it appeared to be the best of the petroleum ether extracts. A fresh "Laughlen" reagent was made (with S6) and left aside for 18 hours before being tested with 167 routine specimens in parallel with the Wassermann and Kahn reactions (Table 24). It is seen that the results are similar to those obtained with S2. Once again the diagnostic specimens reacted with the soya reagent in practically the same way as with Wassermann and Kahn, the most important discrepancies occurring in the treated group. It was noted during this test that although the S6 reagent reacted fairly well, the precipitates which it produced with positive sera were not so clear as those given by the S2 reagent under similar circumstances. Similarly

Table 24.

Results Obtained with Wassermann, Kahn and S6L2 with 167 Routine Specimens.

No.	Wassermann	S6L2	Kahn
	"Diagnostic" Sera		
51	-	-	-
9	++	+++	+++ or ++++
4	-	+	-
5	-	++	++
69			
	"Treated" Sera		
53	-	-	-
7	-	++	++
18	++	++	++
6	++	+	++
4	++	-	++
6	+	++	++
1	±	-	-
3	+	-	++
98			
Total 167			

S6L2 = The second reagent made with extract S6.

the S2 "negatives" were more definite than those given by S6.

Experiments with the Whole Bean:

In parallel with the preparation of the various extracts of soya flour which have just been described, similar extracts were made from the whole soya bean. The bean was reduced to a powder by means of a meat-mincer until a consistency approaching that of the commercial flour was obtained; extracts were made from this "home-made" flour in exactly the same way as described for the extracts S1 to S7. It was confidently expected that these whole bean extracts would produce better results than those made from the flour since the bean contains large amounts of the oil which is rich in phosphatides (Page 69). Some surprise, therefore was felt when the reagents produced from the whole bean extracts showed an apparently complete absence of antigenic activity. All the reagents were "watery" in appearance and gave a granular type of precipitation with every serum tested, both positive and negative. At first this was considered due to some incompatibility in the proportions of the ingredients used in the reagents and each one of the seven extracts was subjected to the alterations which have been referred to previously (Page 80). The extracts were tried at lower and higher concentrations of cholesterol; the diluting saline was altered in respect of quantity used, concentration of NaCl and pH value; the benzoin was tried in different amounts; experiments were made with the extracts concentrated and diluted. None of these manipulations altered the character of the reagents. The results were still uniformly poor; none of the reagents developed the colloidal appearance usually indicative of an active antigen; there was no sign of true aggregation of the stained particles in the presence of strongly positive sera. From these findings it must be concluded that the methods of extraction which give promising results with soya flour are not suitable for use with the whole bean. No evidence of syphilis antigen activity was demonstrated in the whole soya bean by an examination of extracts produced by the methods described.

<u>The Soya Flour Extracts as Antigens for the Wassermann Reaction:</u>

As evidence had been obtained that at least some of the seven extracts made from soya flour possessed a little antigenic activity, an attempt was made to use them as Wassermann antigens. In the Wassermann Test employed in all these investigations (Wyler, 1929 and 1931), the antigen suspension is made up by adding 70 cc. of physiological saline to a mixture consisting of 3 cc. of an alcoholic extract of human heart and 2 cc. of a one per cent alcoholic solution of cholesterol. It was proposed to use the soya extracts in the same way but at the same time to employ weaker and stronger concentrations. Each extract was therefore combined into three

Table 25.

Soya Flour Extracts Used as W.R. Antigens

Serum	W.R.	Kahn	S1 a	S1 b	S1 c	S2 a	S2 b	S2 c	S3 a	S3 b	S3 c	S4 a b c	S5 a b c	S6 a	S6 b	S6 c	S7 a	S7 b	S7 c
1	-	-	-	-	-	-	-	-	-	-	-	Completely Anticomplementary	Completely Anticomplementary	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
8	-	++	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
9	-	++	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
10	±	++	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
11	±	++	-	-	-	-	±	-	-	-	-			-	-	-	-	-	-
12	±	++	-	-	-	-	-	-	-	-	-			-	-	-	-	-	-
13	±	++	-	-	-	-	±	-	-	-	-			-	±	-	-	-	-
14	+	++	-	-	-	-	±	-	-	-	-			-	-	-	-	-	-
15	++	++	-	-	-	+	±	-	-	-	-			-	±	-	-	±	-
16	++	+++	-	±	-	+	+	-	-	-	-			-	±	-	-	±	-
17	++	+++	-	±	-	±	+	-	-	±	-			±	±	-	-	-	-
18	++	+++	±	+	-	+	+	±	-	±	-			±	±	-	±	±	-
19	++	++++	±	+	-	+	+	±	-	-	-			±	+	-	±	±	±
20	++	++++	-	-	-	±	±	-	-	-	-			-	±	-	-	-	-

See text for explanation of "a", "b" and "c".

suspensions made up as follows -

(a) Saline - 70 cc.: one per cent cholesterol - 2 cc.; Extract - 3 cc.

(b) Saline - 70 cc.; one per cent cholesterol - 3 cc.; extract - 5 cc.:

(c) Saline - 70 cc.: one per cent cholesterol - 1 cc. extract - 2 cc.

Using these suspensions as antigens, Wassermann Reactions were performed on 20 selected sera whose responses to the "real" Wassermann and Kahn Tests were known (Table 25). It is obvious from the results that the extracts made poor antigens. S4 and S5 were completely anticomplementary and there was no response whatsoever with S1(c), S3(a)(c) and S6(c). The best results were obtained with S2(b) but even here the amount of complement fixed was, on the whole, small; it will be remembered that S2 was an extract made with hot alcohol after the flour had been thoroughly extracted with ether.

Considering the concentration of extract used to make the suspensions it will be seen that the best results were found with the (b) suspensions, i.e., those with the greatest concentration of extract. This was an indication to try the effect of using the extracts at still higher concentrations but when this was attempted it was found that an anticomplementary zone was entered and that the strength of extract used to make the (b) suspensions was, in fact, practically the upper limit of concentration compatible with a test for complement fixation.

Discussion

The results obtained with the extracts made from soya flour show that the soya bean contains a substance which acts like an antigen in tests for syphilis. The exact nature of this substance is at present unknown but it is probable that it has a structure similar to the syphilis "antigens" found in beef heart extracts and thus may be allied to Pangborn's cardiolipin (Page 95). The determinations of its chemical constitution is beyond the scope of this investigation. The first two extracts may be considered together since their methods of production were so alike. The first extract, which was made in the same way as ordinary Kahn antigen, could be combined as a "Laughlen" reagent which gave reliable results until about 36 hours old when ultrasensitivity developed; this increase in sensitivity developed in the prepared reagent and was not the result of aging in the uncombined extract. The results obtained with the second extract indicate that more antigenic substance is extracted when the ether extractions of the "Kahn" method are followed by extraction with boiling alcohol. When incorporated into a reagent, the second extract certainly produced more definite results and gave a surprisingly good performance when tested with 143 routine specimens in parallel with the Wassermann and Kahn Tests (Table 22). The experiments with these two extracts then, gave support to the contentions of other workers that syphilis antigen may be extracted from plants and in particular indicated that it could be extracted from commercial soya bean flour.

Extract No. 3 did not prove that a better yield of antigen is obtained when the soya flour is treated with acetone before the alcohol extraction nor was there any indication that the antigenic substance was dissolved out beforehand by the acetone. The fourth extract showed that "direct" extraction by alcohol produced poor reagents containing little or no antigen.

Petroleum ether was the chief solvent used in the production of the last three extracts (5, 6 and 7). No. 5 was made by using petroleum ether alone while for No. 6 and No. 7 the soya flour was first treated with ether and acetone, respectively. The three petroleum ether solutions were then evaporated to "syrups" which were dissolved in alcohol; investigation of these alcoholic solutions showed that the best results were obtained with extract No. 6, i.e., the best antigen was produced when the petroleum ether extraction was preceded by treatment with ether.

Of the seven extracts, two gave results definitely superior to the others - Nos. 2 and 6. It is interesting to note that in both of these the soya flour received preliminary treatment with ether before the final extractions were made. On the whole, extract No. 2 gave better results than No. 6 -

the grades of precipitation were more clear-cut and the negatives more easily distinguished by their completely colloidal appearance. Thus the method by which extract No. 2 was produced (ether treatment followed by hot alcohol) apparently gave a higher yield of "antigen" than the other procedures described.

Even with the best of these extracts, hypersensitivity developed within 2 - 3 days of making the "Laughlen" reagents. If the antigenic substances present in the soya flour extracts are (to be considered) the same as those present in extracts of beef heart then this hypersensitivity may be presumed to be due to some unknown factor peculiar to the soya flour: the removal or control of this factor is necessary before the antigenic substances in soya flour can be used with advantage.

The poor results obtained with extracts made from the whole bean are surprising in view of the fact that the bean contains a high percentage of an oil said to be rich in phosphatides. Presumably the oil interferes with the extraction of the antigenic principle or some component of the oil may pass across into the extract and prevent the formation of a sensitive emulsion on the addition of saline. This last suggestion is supported by the results of an experiment in which various oils, both vegetable and mineral, were added to different batches of Kahn antigen; Laughlen reagents made from these modified Kahn antigens had the same characteristics as those made with extracts of the whole soya bean - they were watery in appearance and gave granular precipitation with all sera. There is, of course, the probability that none of the extraction methods employed was suitable for use with the whole bean; it is felt that this problem is worthy of further investigation since it is almost certain that, given a suitable method, the oil-containing bean would yield better antigens for syphilis than those given by the flour from which the greater part of the oil has been removed.

Attempts to use the flour extracts as antigens for the Wassermann Reaction were largely unsuccessful but two points of interest were demonstrated - (a) an antigenic response, though weak, was presented by at least three of the extracts: and (b) this response was most marked with extracts Nos. 2 and 6. (It will be remembered that these two extracts also gave the best results with the precipitation tests.)

Summary

It has been demonstrated that the soya bean contains a substance which will react with syphilitic sera in the same way as other syphilis "antigens". The activity of this substance, extracted by the methods described, is much less than that of the antigenic principle of beef heart and reagents containing it show a tendency to become oversensitive within a few days. The best results were obtained with extracts of commercial soya flour and the best method of extraction was to treat the flour with four changes of ether (as for the Kahn antigen) before extracting with boiling alcohol. Extracts made from crushed whole bean displayed no antigenic activity. The modified Laughlen Test was found very suitable for investigating the antigenic properties of the numerous extracts prepared.

(b) The Significance of the Diaminomonophosphatide Sphingomyelin.

The exact chemical constitution of syphilis antigen is still largely a mystery but from the earliest days of the Wassermann Reaction it has been known that the active principle has a structure similar to the phosphatides (phospholipids). Many attempts have been made to identify the phosphatides (lecithin, cephalin and sphingomyelin) with this active principle and much contradictory evidence has been produced. It is obvious from the large volume of literature on the subject that one of the main difficulties has been that the phosphatides are not easily obtained in the pure state and that many workers in the past have reported results which they believed were due to one particular substance which in fact consisted of a mixture. Early work favoured lecithin as the responsible agent (Page 11) but more recent reports indicate that <u>pure</u> lecithin has no antigenic activity (Maclean, 1927, Wadsworth, Maltaner and Maltaner, 1935, and Pangborn, 1944).

In 1937, Eagle gave the following reasons for the belief that the phosphatides and syphilis antigen are closely linked -

(a) The active part of the antigen is soluble in fat solvents such as alcohol and ether and is insoluble in water:

(b) It is relatively insoluble in acetone and is insoluble in cadmium chloride:

(c) It is still active after heating to 120° Centigrade but is destroyed by strong alkalis and acids; and

(d) Acid hydrolysis produces a base, a phosphate, glycerol and ammonium salts.

These properties are practically identical with those of lecithin. Eagle added the further observation that the nitrogen content of the active principle was similar to that of lecithin.

Until recently then, syphilis antigen was believed to be "some kind of phosphatide", presumably akin to the three known members of that group, but not exactly definable in the chemical sense. Nothing more definite was known until 1941 when Pangborn reported the isolation of a new phosphatide which she called Cardiolipin. This substance had been prepared from an alcoholic extract of beef heart and was stated to be "serologically active". In a series of publications (Pangborn 1941, 1942, 1944, 1945 and 1947), simplified methods of obtaining this cardiolipin were described and a tentative formula proposed -

$CH_2OR.CHOR.CH_2O.P.OCH_2CHORCH_2O.P.OCH_2CHOR\ CH_2O.P.OCH_2CHOR.CH\ OR$

O ONa O ONa O ONa

in which R = fatty acid. Briefly, the method of extracting cardiolipin (Pangborn, 1945) is to treat fresh minced beef heart with acetone, dry the tissue, powder it and extract with methyl alcohol: then precipitate out crude cardiolipin salts by means of barium chloride which leaves lecithin still in solution in the alcohol. The cardiolipin salts are now converted to sodium salts and purified by treating with acetone and ether. Further purification, including conversion to a cadmium salt, follows, the final product emerging as pure sodium cardiolipin.

Pangborn (1947) describes cardiolipin as a complex phosphatidic acid. In contrast to what was held previously concerning syphilis antigens, cardiolipin contains no nitrogen but is similar in that it contains fatty acids and phosphorus. Regarding its antigenic activity there are some curious features. Together with pure lecithin and cholesterol it forms what may be termed an antigenic triad which must be intact in order to exert full activity as an antigen, i.e., these three substances are largely interdependent and any two of them will not satisfactorily perform the functions of syphilis antigen without the presence of the third. Thus, cardiolipin will not fix complement (in the presence of syphilitic sera) if cholesterol is absent and is strongly anticomplementary in the absence of lecithin. From this evidence it would seem that there is no single substance which can act as an "antigen" to syphilis and that the reports in the past concerning the use of lecithin as an antigen must have owed their good results to the fact that the lecithin employed was an impure mixture containing cardiolipin or some similar substance.

The practical value of cardiolipin has been illustrated by its use along with lecithin and cholesterol as an antigen for syphilis in both complement-fixation reactions and precipitation tests (Brown, 1944; Harris and Portnoy, 1944; Maltaner and Maltaner, 1945; and Vogelsang, 1948) and from the results reported there seems to be no doubt that a combination of these three substances provides an efficient antigen. This is the first definite indication that it is possible to prepare syphilis antigen from known chemical entities and in the light of this recent work the chemical structure of syphilis antigen may be said to consist of two main components, pure lecithin and pure cardiolipin, the combined activity of which is enhanced by the presence of cholesterol.

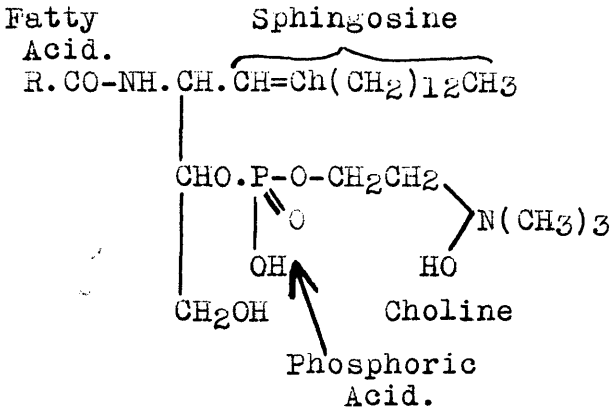
In 1945 there appeared a report on the purification of syphilis antigen which seemed to contradict some of the claims made for cardiolipin (Fischer, Fischer-Dallmann and Boné). This report described an antigen which had been prepared from beef heart extract by adsorbing the extract with aluminium hydroxide, eluting with benzene and purifying with hot alcohol and petroleum ether. An exact formula for the substance obtained was not given but it was stated to contain only a trace of phosphorus (probably an impurity), 0.25 per cent glucose and 40 per cent fatty acids. Serologically, this compound could be used as an antigen for syphilis without the addition of lecithin or cholesterol and it was not anti-complementary At first sight one would say that the obvious answer is that this "antigen" was not pure chemically and indeed Fischer and his co-workers themselves suggest that it may contain some cardiolipin, but it is difficult to explain the virtual absence of phosphorus. There is always the possibility, of course, that more than one substance can act as an antigen to syphilis <u>in vitro</u>.

The general reliability and serological flexibility of the chemically undefined meat extracts now universally used are probably much superior to any pure chemical combination so far prepared; the factor of major importance in such extracts is undoubtedly related to the phosphatides but has not so far been identified with any certainty. It seems desirable therefore to continue to investigate the antigenic activities of all known phosphatides. Sphingomyelin has received but little attention in this field and is the subject of the present investigation.

The information which prompted the present investigation was a report by Neymann and Gager (1917) who found that the most highly antigenic constituent of tissue extracts used as antigens in tests for syphilis was a diaminomonophosphatide. As it is generally accepted (Page 101) that sphingomyelin is the only diaminomonophosphatide, the precise identity of syphilis antigen seems to have been revealed, but the evidence of more modern work suggests that Neymann and Gager's fraction may not have been absolutely pure and may have been a mixture containing say some "cardiolipin". Nevertheless it is probable that the greater part of their extract consisted of a diaminomonophosphatide and thus sphingomyelin is indicated as the possible antigenic agent present. Further, as previously mentioned (Page 17) acetone and ether are employed frequently in the "purification" of antigens used in precipitation tests for syphilis; two instances of this are the manufacture of the antigens for the Kahn and Kline Tests in which, respectively, the ether and acetone extracts of beef heart lipids are discarded, only the alcohol-soluble portions being used in the actual antigen. Now, if it is to be assumed that the antigenic substance is the same in both these antigens it follows that this substance must be at least relatively insoluble in both ether and acetone. Additional evidence of this is the fact that Pangborn (1941, 1944 and 1945) "purified" her antigenic substances (cardiolipin) by washing with ether and acetone, a procedure which indicates again the insolubility of the antigen in these liquids. As has been described (Page 94) all the phosphatides are relatively insoluble in acetone but one differentiating feature of sphingomyelin is that it is also practically insoluble in ether, cold or hot (Maclean and Maclean, 1927), whereas both lecithin and cephalin are easily soluble in ether, especially if warmed. Thus it would seem that sphingomeylin is the only phosphatide which could be left undissolved in a tissue treated with acetone and ether and when dissolved out in a subsequent alcoholic extraction of the tissue it would constitute at least a part of an antigen made from such an extract. In contrast to this it is of interest to note a report by Brown and Kolmer (1941), who concluded that the ether-insoluble alcohol-soluble fraction of Kahn antigen was completely inactive. It is difficult to say whether this fraction would be the same as the ether-insoluble alcohol-soluble extract (? sphingomyelin) of dried beef heart since such an extract would itself constitute Kahn antigen, but it is obvious that so many solubilities and insolubilities are implicated in dealing with these lipoidal substances that the exact definition of their individual properties is not easy. Even allowing for these difficulties, however, it seemed likely that an investigation of the antigenic properties of sphingomyelin might yield information of some value.

A Note on the Chemistry of Sphingomyelin:

Chemically, sphingomyelin is described as a diaminomono-phosphatide and is a member of a group of organic phosphorus compounds known variously as phosphatides, phospholipins, lipids or "lecithins". Two other phosphatides are known to exist with certainty; these are lecithin and cephalin, both of which are monoaminomonophosphatides and differ from sphingomyelin in that they contain glycerol. The formula for sphingomyelin shows it to be a relatively complex substance:-



(R = Fatty Acid)

In the pure state it consists of white or pale yellow crystals, "feathery" in appearance and powdery to the touch. Like the other phosphatides, sphingomyelin is soluble in many organic solvents but unlike them it is almost insoluble in cold alcohol and cold ether; all the phosphatides are insoluble in acetone. (Maclean, 1927; Thannhauser, 1940; and Thorpe, 1947).

The Method of Extraction:

The richest source of the phosphatides is nervous tissue (Thorpe, 1947) and thus the human brain was considered suitable for the extraction of sphingomyelin. The composition of nervous tissue varies according to situation but all types have two features in common; they have a high phosphatide content and they contain large amounts of cholesterol. Quoting Koch, Thorpe (1947) gives the constituents of brain as follows -

	White Matter	Grey Matter
Water	70%	84%
Protein	10%	8%
Phosphatides	8.5%	3.7%
Cerebrosides	5%	3%
Cholesterol	5%	0.7%
Inorganic Material	0.8%	0.8%

It has been abundantly demonstrated that the amount of phosphatide obtainable from dried tissues is about four times the quantity which can be extracted when the tissues are wet; varying results have been reported by many investigators but those of Mayer and Schaeffer (1914) may be taken as being average examples -

	Dried Tissue		Wet Tissue	
Rabbit	Phosphorus (Per Cent)	Lecithin (Per Cent)	Phosphorus (Per Cent)	Lecithin (Per Cent)
Liver	0.54	13.5	0.142	3.54
Kidney	0.54	13.5	0.122	3.05
Lung	0.49	12.2	0.096	2.40
Muscle	0.17	4.2	0.039	0.98

In order to obtain the best yield, therefore, it was necessary to dry the brain tissue before attempting to extract phosphatides. There are several methods of drying but owing to the ease with which the phosphatides decompose certain precautions must be observed, e.g., the drying must be carried out at low temperatures: it must be completed as quickly as possible; and exposure to air during the process must be minimal. Consideration of these conditions led to the following method being employed - two human brains were obtained as soon after death as possible and thoroughly rinsed in cold water to remove gross blood contamination; they were then cut up into small cubes about a quarter-of-an-inch square and again rinsed in water. The tissue was next pounded to a thick paste in a mortar and placed in a 3-litre flask containing 500 cc. acetone.

((Treatment with acetone at this stage has several advantages. A large amount of water is absorbed from the material and fat, fatty acids and cholesterol are to some extent removed. Being insoluble in acetone, the phosphatides are left intact though there is some evidence that they are sparingly soluble in acetone in which fatty substances are dissolved (Erlandsen, 1907, quoted by MacLean, 1927). When the process is kept at low temperatures the amount removed is small.))

It was allowed to stand at 4^{o} Centigrade for 30 minutes then the acetone was poured off and a further 500 cc. added to the flask which was shaken and left for another 30-minute period at 4° Centigrade. The mixture was now filtered, the brain pulp being well pressed between layers of lint to remove as much liquid as possible. Shallow dishes containing the pulp were placed in dessicators containing fresh quick-lime. By the application of a suction pump for 45 minutes, the air was evacuated from the dessicators which were then sealed and placed in an ice-chest at 4° Centigrade. At the end of 48 hours the tissue was found to have caked into hard layers of dry pale yellow greasy-looking material. Broken up and ground to a coarse powder in a mortar, the dry tissue was found to weigh 425 grammes. To reduce the material to as fine a powder as possible, further grinding was carried out by means of a meat-mincing machine fitted with small-mesh cutters.

There are several methods of extracting sphingomyelin from dried tissue but in general the principles are similar. Levene (1916) described one by which he believed sphingomyelin could be obtained in the pure state; this was a modification of a method he had first published in 1914. That his method gave good results is evidenced by the fact that it is still used at the present time (Thannhauser, Benotti and Bonocoddo, 1946, and Tompkins, 1946).

When sphingomyelin is isolated from brain tissue it requires to be separated from the substances with which it is invariably accompanied - lecithin, cephalin and galactolipins (sugar-containing, phosphorous-free lipoids). Treatment with alcohol and ether will remove the lecithin and cephalin and the galactolipins can be separated off by means of the organic solvent pyridine in which sphingomyelin is practically insoluble when cold but easily soluble when hot (Rosenheim and Tebb, 1910).

Following Levene's method, 100 gm. of the dried brain tissue was placed in a 500 cc. flask with 200 cc. of the absolute ethyl alcohol. The flask was placed in a water-bath at 85° Centigrade and the contents allowed to boil for 30 minutes when the alcohol was poured off into another flask. The tissue was again extracted with the same amount of alcohol twice, the three extracts being pooled. This hot alcoholic extract contained lecithin, cephalin, sphingomyelin and galactolipins, but when cooled by leaving overnight at 4° Centigrade a heavy white precipitate settled out. This precipitate contained the sphingomyelin and galactolipins since these two substances are practically insoluble in cold alcohol; it was separated by filtering through Whatman's No. 1 paper and dried on the filter paper in a 37° Centigrade incubator. When dry the precipitate was seen to consist of pale yellow soft feathery crystals, slightly oily to touch. To remove any traces of lecithin, cephalin and unwanted fatty substances, the crystals were shaken up with 100 cc. of pure ether, filtered and dried and similarly treated with 100 cc. of pure acetone. When dry and free from the odour of acetone the crystals, now more powdery and about 4.0 gm. in weight, were dissolved in 200 cc. of hot "technical" pyridine, boiled for a few minutes then cooled by leaving the solution overnight at 4° Centigrade. The next morning a cloudy precipitate of crude sphingomyelin was seen which, by reason of its insolubility in cold pyridine, had separated out leaving the galactolipins in solution. The precipitate was filtered off and dried and dissolved in 50 cc. of hot glacial acetic acid; on cooling, a slight deposit (a mixture of impurities) appeared and was removed by centrifuging. The acid solution containing the sphingomyelin was now concentrated by placing it in a dessicator which was evacuated by means of a Hyvac pump. When reduced to about 30 cc. it was removed from the dessicator and poured into 100 cc. of pure acetone: the sphingomyelin, being insoluble, was at once precipitated out in the form of a cloud of very fine scintillating particles - this is stated by Levene to be "crude sphingomyelin". The remaining steps of Levene's method are concerned with the purification of the product obtained at this stage.

The precipitate was filtered clear of the acetone and was found to consist of a fine cream-coloured powdery substance - 3.7 gm. were obtained. This was dried and dissolved in 50 cc. of a mixture of petroleum ether and ethyl alcohol (5 : 1) and to this solution was added a little 98 per cent alcohol which caused the appearance of a fine cloudiness in the liquid. The 98 per cent alcohol was added (a total of 12.5 cc. was used) until no more precipitate was formed; then the liquid was filtered twice to remove this presumed lipoid impurity. Concentration of the filtrate was next carried out in a dessicator as before and when reduced to approximately half its original volume it was poured into 100 cc. of pure acetone and left at 4° Centigrade for six hours. As previously, the addition of the concentrated filtrate to the acetone caused the immediate precipitation of fine white crystals of sphingomyelin and at the end of an empirical six-hour period this precipitate was separated by filtration, dried and dissolved in 25 cc. of a mixture consisting of equal parts of pyridine and pure chloroform at 60° Centigrade. After this solution had cooled and evaporated at room temperature for 12 hours a crystalline deposit formed round the sides of the containing beaker; evaporation was allowed to continue until only about 10 - 12 cc. of the mother liquor was left; this was poured away and the beaker was placed in an incubator at 37° Centigrade for several hours. When the crystals were completely dry they were carefully scraped on to filter paper and weighed; 3.16 gm. were obtained. This crystallization from a warm mixture of pyridine and chloroform is a slightly modified version of Levene's method which involves several recrystallizations from pyridine and chloroform at room temperature, at 30° Centigrade and at 37° Centigrade. For the purposes of the present investigation, however, the sphingomyelin obtained as described was considered suitable.

Investigation

(a) To discover whether this sample of sphingomyelin could be used *per se* as an antigen for the diagnosis of syphilis.

(b) To assess the effect of its addition to a known syphilis antigen.

The insolubility of sphingomyelin in cold ethyl alcohol meant that it could not be made up as a Wassermann or Kahn antigen according to usual technique but it could be made up as a "Laughlen" reagent since this is made at a temperature of 50° Centigrade. The experience gained with the modified version of the Laughlen Test (Section One) made it particularly suitable for the purposes of this investigation.

(a) Sphingomyelin Used Alone as an Antigen:

A one per cent solution of the crude sphingomyelin was made by adding 0.1 gm. to 10 cc. of absolute ethyl alcohol in a 6" x $\frac{5}{8}$" Pyrex test tube. As expected, the sphingomyelin did not dissolve in the alcohol at room temperature but solution was obtained when the mixture was warmed to 40° Centigrade in a water-bath and shaken vigorously. The tube was then stoppered tightly with foil-covered cotton-wool and stored at 37° Centigrade in an incubator. Using this solution in place of Kahn antigen a "Laughlen" reagent was made as described in Section One (Page 36), the only difference in procedure being that whereas the Kahn antigen is mixed with the stain and benzoin in the cold and then brought to 50° Centigrade the sphingomyelin solution was, of necessity, kept at this temperature from the beginning, the amount required (1 cc.) to make the reagent being taken from the stock solution stored at 37° Centigrade and placed in another Pyrex tube which was immediately transferred to the 50° Centigrade water-bath. The stain and the benzoin were added while the sphingomyelin was at this temperature, i.e., without removing the tube from the bath. The remainder of the technique was as described in Section One.

When the buffered saline was poured into the mixture of sphingomyelin, stain and benzoin a "pink milk" liquid was produced exactly similar in appearance to that obtained when using Kahn antigen and thus at first sight it seemed that the sphingomyelin had supplied the lipoid material necessary for the production of a smoothly colloidal Laughlen reagent. When the modified reagent had been allowed to stand for two hours, however, it was found that a coarse granularity had appeared and that some of the "granules" had coalesced into clumps which had floated to the surface of the liquid, leaving a pale-pink watery-looking fluid beneath. Vigorous shaking apparently restored the former colloidal appearance but within 30 minutes the granularity was again present. It was obvious that at this stage the reagent was useless for testing. Tests would have to be made before the granularity had

Table 26.

The Antigenic Properties of a One Per Cent Sphingomyelin Solution.

Serum	W.R.	Kahn	S.R. 20 Minutes Old	S.R. 1 Hour Old	S.R. 2 Hours Old	M.L.
1	-	-	Tr.	Tr.	Tr.	-
2	-	-	-	Tr.	Tr.	-
3	-	-	Tr.	Tr.	Tr.	-
4	-	-	-	-	Tr.	-
5	-	-	-	-	Tr.	-
6	++	++	Tr.	Tr.	Tr.	+++
7	++	+++	+	+	+	+++
8	++	+++	Tr.	+	+	+++
9	++	+++	Tr.	Tr.	Tr.	+++
10	++	++++	Tr.	Tr.	Tr.	++++

S.R. = Reagent made with one per cent sphingomyelin.

M.L. = An ordinary modified Laughlen reagent made with Kahn antigen.

Tr. = Trace of precipitation.

appeared, i.e., shortly after the reagent had been made.

Accordingly, a second reagent was made exactly as before and having been allowed to stand only 20 minutes its reactivity was tested with ten sera of known response to the Wassermann and Kahn reactions (Table 26). It is seen that at this point the reagent was for practical purposes devoid of activity. Tested again when one hour and two hours old, there was no indication of the development of any sensitivity to the positive sera but, as will be seen from the Table, only an increased tendency to a granularity which produced faintly positive readings with all sera. As with the first reagent, massive granularity began to appear soon after this and further testing was impossible.

This first experiment suggested that the lipoid content of a one per cent solution of crude sphingomyelin was sufficient to form but not enough to maintain the colloidal state required in a reagent of the Laughlen type. A stronger solution was therefore tried. This was obtained by concentrating the stock solution; two per cent was the required strength; 2 cc. of the stock solution was allowed to evaporate at 37° Centigrade until reduced to exactly 1 cc. and, using this in place of the Kahn antigen, a "Laughlen" reagent was prepared as before. Again this reagent had initially the smooth colloidal appearance of the normal Laughlen reagent: tests were begun 15 minutes after manufacture and continued at

Table 27.

The Antigenic Properties of a Two Per Cent Solution of Crude Sphingomyelin.

Serum	W.R.	Kahn	Age of "Sphingomyelin Reagent": 15 Minutes	45 Minutes	One Hour	Two Hours	Three Hours	Four Hours	M.L.
1	-	-	-	-	-	Tr.	Tr.	All tests granular. No readings possible.	-
2	-	-	-	-	-	-	-		-
3	-	-	-	Tr.	Tr.	Tr.	Tr.		-
4	-	-	-	-	Tr.	Tr.	Tr.		-
5	-	-	-	Tr.	Tr.	Tr.	Tr.		-
6	++	++	-	Tr.	Tr.	Tr.	Tr.		+++
7	++	+++	-	Tr.	Tr.	Tr.	Tr.		+++
8	++	+++	-	-	-	-	-		+++
9	++	++++	-	-	-	-	Tr.		++++
10	++	++++	-	-	Tr.	Tr.	Tr.		++++

M.L. = An ordinary modified Laughlen reagent made with Kahn antigen.

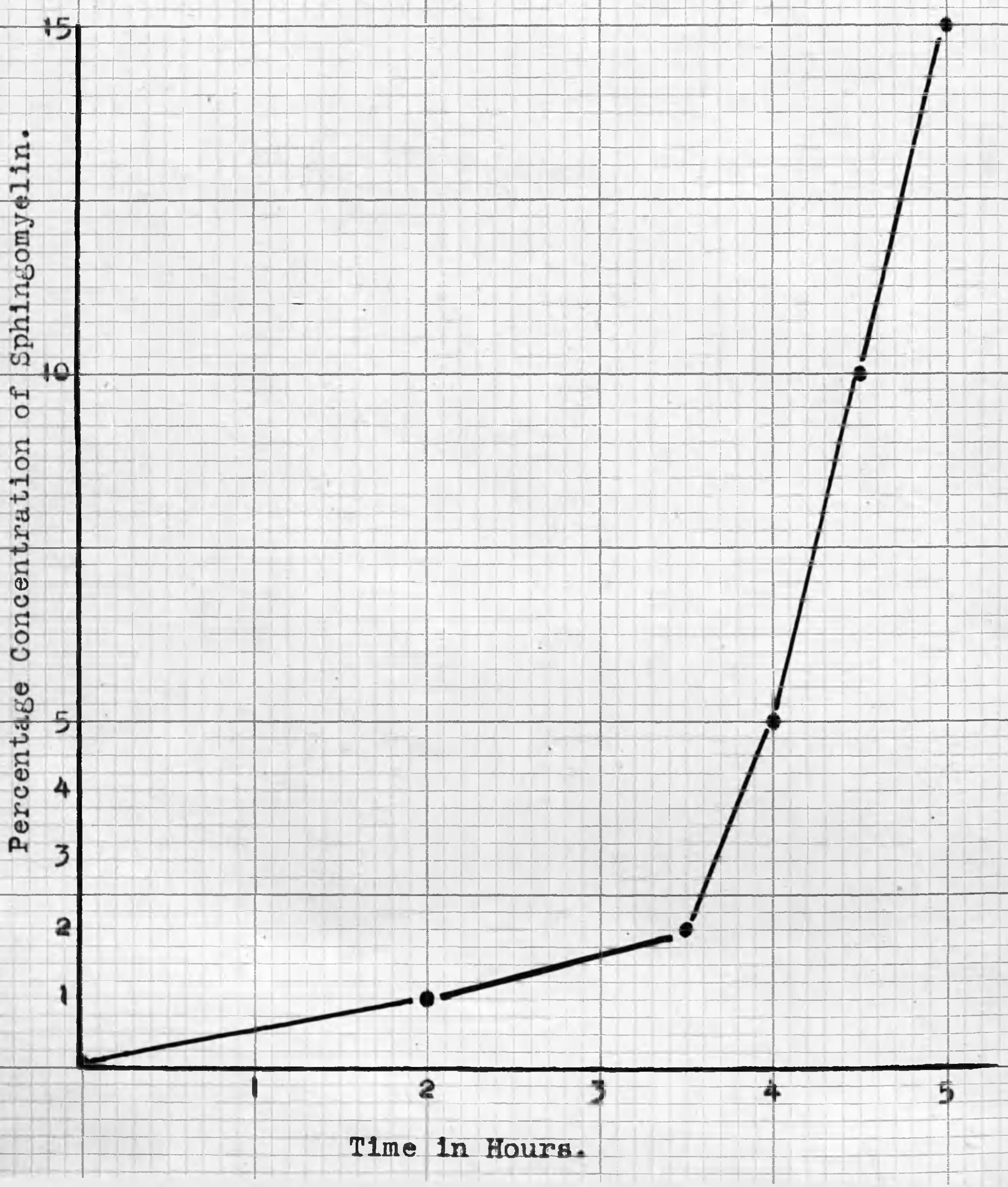
Tr. = A trace of precipitation.

intervals until the reagent was four hours old (Table 27). It will be seen once more that there was no evidence that the reagent was in the least sensitive to syphilitic sera and, as with the one per cent reagent, the only finding was that a granularity progressively developed until when about three-and-a-half hours old the reagent was unusable. Half-an-hour later the massive clumping of the "granules" was fully developed; the colloidal appearance could be restored as previously by shaking but within 40 - 45 minutes the clumping of the stained particles was again present. The only difference between the reagent made with one per cent and that made with two per cent sphingomyelin was that this spontaneous coalescence of suspended particles did not develop so rapidly with the latter and no doubt this can be explained by the increase in available lipoid; this increase, however, is still not sufficient to maintain the colloidal state of the reagent for more than three to four hours.

Greater concentrations of sphingomyelin were now employed - 5 per cent, 10 per cent and 15 per cent - but tests with the reagents produced did not give any evidence of the presence of syphilis antigen. The massive clumping referred to above developed in all three reagents when four hours old with the five per cent solution, four-and-a-half with the ten per cent and five hours with the 15 per cent. A concentration greater than 15 per cent was not possible as this appeared to be the

Figure 6.

The Onset of Spontaneous Massive Coalescence of the Stained Particles in the Laughlen-Type Reagents Made With Various Concentrations of Sphingomyelin.



limit of solubility of the sphingomyelin in alcohol at 50° Centigrade - even the 15 per cent strength was obtained only with some difficulty after prolonged shaking. Concentrations below one per cent were also tried with negative results; the reduction in lipid content produced watery-looking reagents with little or no colloidal appearance even immediately after being made. A control reagent made at this point, using cholesterolised alcohol only in place of Kahn antigen, had no colloidal qualities whatsoever and the stained particles coalesced into clumps as soon as the dilution was made with the buffer-saline. The times of onset of this spontaneous coalescence in the various reagents have been represented graphically in Figure 6.

The possibility that an antigenic property of the sphingomyelin had been masked in some way by the other constituents of the Laughlen reagent was next investigated. Reagents were made with the five concentrations of sphingomyelin (1 - 15 per cent) as before and with each concentration the following variations were made in the constitution of the reagent -

(a) The diluting saline was altered to 1.5 per cent and 5 per cent;

(b) The pH value of the diluting saline was altered to 8;

(c) The amount of benzoin added to 1 cc. of sphingomyelin was tried at 0.15 cc. and 0.6 cc.; and

(d) Temperatures of 37° Centigrade and 65° Centigrade were used in place of the usual 50° Centigrade.

The foregoing variations gave rise to a large number of combinations of the different constituents but in not one of the reagents produced thereby was there found any evidence of syphilis antigen activity. Those made with the stronger saline gave results similar to what had been obtained already and no noticable difference was produced by the pH value alteration; the raising and lowering of the benzoin content merely gave rise to "thicker" and "thinner" reagents and temperatures below and above the usual 50° Centigrade only delayed and hastened respectively the onset of "granularity".

(b) <u>The Effect of Adding Sphingomyelin to a Known Syphilis Antigen</u>:

The next object of the investigation was an estimation of the effect of adding crude sphingomyelin to a known syphilis antigen. It was convenient to study the effect produced on Kahn antigen since this normally forms the basis of the modified Laughlen reagent. Twelve solutions of sphingomyelin were prepared with concentrations of 1, 2, 3, etc., up to 10 and 12 and 15 per cent. It was proposed to add 0.05 cc. of each concentration to 1 cc. of Kahn antigen and to test the reactions of the modified Laughlen reagents produced from these mixtures, controlled by a reagent made from 1 cc. of Kahn antigen to which had been added 0.05 cc. of cholesterolised

Table 28

Initial Test of Modified Laughlen Reagents Made with Kahn Antigen Plus Varying Amounts of Sphingomyelin. Fifty Selected Sera from "Diagnostic" Cases.

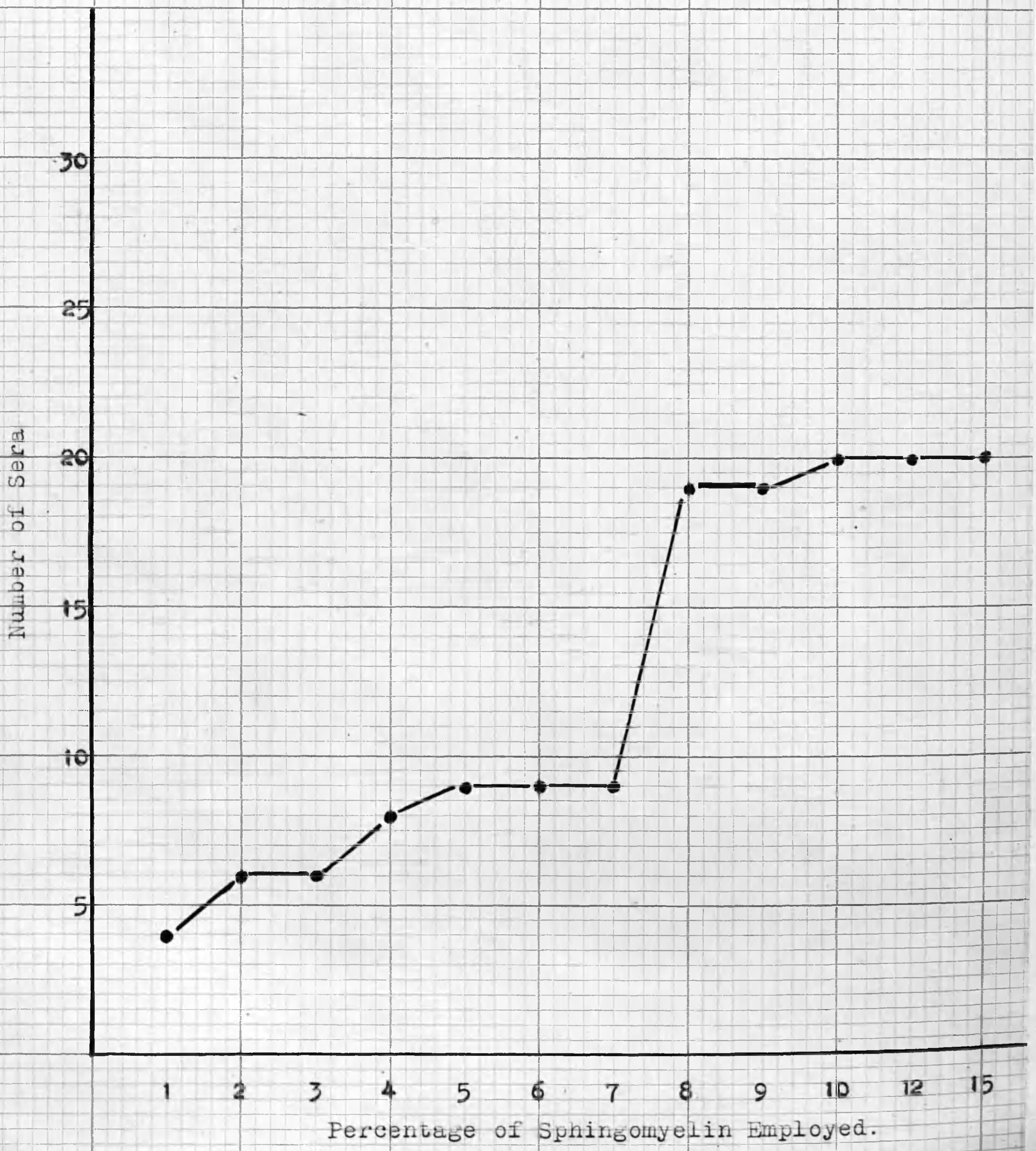
Serum	W.R.	Kahn	M.L.	M.L.^{2}	Percentage of Sphingomyelin											
					1	2	3	4	5	6	7	8	9	10	12	15
1	++	++++	++++	++++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
2	++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
3	++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
4	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
5	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
6	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
7	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
8	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++	++++	++++	++++	++++	++++
9	++	++	+++	+++	+++	+++	+++	+++	+++	+++	++++	++++	++++	++++	++++	++++
10	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++	++++	++++	++++	++++
11	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
12	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
13	+	++	++	++	++	++	++	++	++	++	++	+++	+++	+++	+++	+++
14	+	++	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
15	+	++	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
16	±	++	+	+	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
17	±	++	++	++	++	++	++	++	++	++	++	+++	+++	+++	+++	+++
18	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
19	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
20	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
21	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
22	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
23	−	−	−	−	+	+	+	+	+	++	++	++	++	++	++	++
24	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+
25	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
26	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
27	−	−	+	+	+	+	+	+	+	+	++	++	++	++	++	++
28	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
30	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
31	−	−	+	+	+	+	+	+	++	++	++	++	++	++	++	++
32	−	−	−	−	−	+	+	+	+	+	+	++	++	++	++	++
33	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
34	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
35	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
36	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
37	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
38	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
39	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
40	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
41	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
42	−	−	−	−	−	+	+	+	+	++	++	++	++	++	++	++
43	−	−	−	−	−	−	−	+	+	+	+	++	++	++	++	++
44	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
45	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
46	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
47	−	−	−	−	−	−	−	−	+	+	+	++	++	++	++	++
48	−	−	−	−	−	−	−	+	+	+	+	++	++	++	++	++
49	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
50	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+

alcohol. In this connection it will be remembered (Page 28) that when the maximum amount of stain solution was being considered it was found that up to 0.15 cc. of alcohol could be added to 1 cc. of Kahn antigen without upsetting the properties of a Laughlen reagent made with this diluted antigen as its basis; of this 0.15 cc. quantity, 0.1 cc. is now taken up by the stain solution leaving "available" 0.05 cc. Thus it was considered that this amount of sphingomyelin solution could be added to the Kahn antigen without causing undue complication in the interpretation of results.

Reagents were prepared from the 12 quantities of diluted Kahn antigen and from the sample diluted with alcohol which was to serve as a control and tests were carried out, after the usual 36 hours "ripening" period, with 50 selected sera from untreated cases in parallel with the Wassermann, Kahn and modified Laughlen tests (Table 28). From the results obtained it is seen that the control reagent (M.L.^{2}) reacted in exactly the same way as the ordinary modified Laughlen reagent and was thus an excellent standard of comparison. The "sphingomyelin reagents" gave evidence of the development of hypersensitivity which increased in direct proportion to the percentage of sphingomyelin present. The one per cent sphingomyelin reagent produced results similar to those produced by the control reagent but with the higher concentrations various degrees of precipitation began to appear

Figure 7.

The Number of Non-Specific Positive Reactions Occurring with Modified Laughlen Reagents Containing Different Amounts of Sphingomyelin.



with sera which were almost certainly non-syphilitic. An attempt to represent this increase in sensitivity is shown in Figure 7. This graph gives the number of presumably non-syphilitic sera (Nos. 21 - 50, inclusive) which produced some degree of precipitation with the different sphingomyelin reagents. It is seen that the graph rises at a level between four and five per cent and again, more steeply, at eight per cent; these features seem to indicate that the addition of sphingomyelin to the Kann antigen basis of the Laughlen reagent produces an increase in sensitivity giving rise to false positive reactions, the number of which tends to increase at concentrations of 0.05 cc. of four per cent and (more sharply) eight per cent sphingomyelin per 1 cc. Kahn antigen. An examination of the results produced by the "positive" sera is interesting because it shows that no case of syphilis was missed by the sphingomyelin reagents and closer scrutiny reveals that several times an increase in the degree of precipitation occurred with the reagents containing 0.05 cc. of five and eight per cent sphingomyelin. This again points to an increase in sensitivity occurring with these two strengths as previously indicated in Figure 7.

To estimate the effect of age on the reagents containing sphingomyelin, they were tested at intervals over a period of four weeks. (It will be remembered that they were already 36 hours old when used in the investigation represented in

Table 28.) As was expected an increasing sensitivity was found. This was established in the higher concentrations first and by the end of seven days from the time of manufacture the reagents made from Kahn antigen diluted with the 8, 9, 10, 12 and 15 per cent strengths of sphingomyelin were producing strongly positive precipitation of a granular character with all sera, positive or negative. The process was continuous and all the reagents gradually assumed this hypersensitivity in descending order of sphingomyelin concentration. Twenty-one days after manufacture granular precipitation was noticed in the "one per cent" reagent which up to this point had maintained close similarity with the results obtained with the control, M.L.[2].

Referring to Table 28, it will be seen that certain sera (23, 30, 32, 42, 45, 47 and 48) reacted negatively with Wassermann, Kahn and modified Laughlen and with the control reagent but had produced precipitation with practically all the sphingomyelin reagents. Since these sera were all from "diagnostic" cases, there was the possibility that the sphingomyelin reagents had detected syphilis earlier than the other tests. Thus, on the basis of similar tests which were described in Section One, this possibility was investigated by a re-examination of these cases after an interval of three weeks from the time of the first testing. All proved to be negative to the Wassermann, Kahn Test and modified Laughlen and it was therefore concluded that the reactions produced earlier by the sphingomyelin reagents were purely non-specific.

Discussion

The phosphatide extract used in this investigation has been called "crude" sphingomyelin. Nevertheless that it was at least reasonably pure is supported by two circumstances -

(a) It was extracted by an accepted standard procedure; and

(b) Some comparative experiments with a sample of pure sphingomyelin supplied by Dr. O. Rosenheim, National Institute for Medical Research, London, gave results similar to those obtained with the "crude" product.

It is therefore submitted that the findings of this investigation are a measure of the antigenic potentialities of sphingomyelin for syphilis.

From the results of the tests made with the reagents which contained only a solution of sphingomyelin as their basic constituent it seems that the activity of syphilis "antigens" cannot be ascribed to this member of the phosphatide group. Before the investigation was undertaken, the evidence in favour of sphingomyelin being the responsible agent was quite impressive, but it can only be concluded that once again the confusion which apparently exists even at the present time concerning the solubilities and other properties of the phosphatide compounds caused erroneous deductions. For example, the "highly antigenic" diaminomonophosphatide of Neymann and Gager can hardly have been the same substance as that used in the present work; yet the latter compound was almost certainly a diaminomonophosphatide, being presumably mainly composed of sphingomyelin though it gave no indication of possessing any activity whatsoever as a syphilis antigen.

The effect of adding crude sphingomyelin to a known syphilis antigen is quite clearly demonstrated by the more positive findings of the second part of the investigation. It is shown that this addition results in an immediate increase in sensitivity with the production of non-specific positives. There is no evidence that this increase in sensitivity is of any value in the earlier diagnosis of syphilis though the addition of the smallest amounts of sphingomyelin seemed to have an enhancing effect on the degrees of precipitation produced. It is thought that this sensitising effect of sphingomyelin may be of some importance and might indicate that this phosphatide is linked with syphilis antigen in a manner similar to that of lecithin with cardiolipin (Page 96), i.e., that this linkage is in some way necessary for the development of full sensitivity. Bearing upon this it is to be remembered that sphingomyelin is almost certainly present in many syphilis antigens; the method of preparing Kahn antigen, for example, leaves little doubt that some sphingomyelin is contained in the final extract (vide Page 100). The assessment of the full significance of the effect of sphingomyelin on syphilis antigen activity falls largely within the field of the biochemist as has been the case with lecithin and cephalin and with the development of the use of cardiolipin.

Summary

A sample of crude sphingomyelin, prepared by a standard method, has been examined in an attempt to estimate its effect on the activity of syphilis antigen. Because sphingomyelin is soluble in alcohol only when hot, the modified Laughlen Test, the reagent for which is manufactured at a temperature of 50° Centigrade, was particularly suitable as a method of research. The results obtained show that sphingomyelin cannot be used alone as a syphilis antigen but that it has the effect of increasing the sensitivity of known antigens. This sensitivity intensifies the degree of precipitation obtained but increases the number of non-specific results.

General Summary

It is probably true to say that the merits of the precipitation tests for syphilis depend more on their particular methods of performance than on the intrinsic qualities of their individual antigens, which are all similar and in many cases identical. Thus the value of the Laughlen Test will depend upon whether its method of performance is convenient for the conditions under which the test is to be used. The results obtained in Section One of this thesis have shown that the present modification of the Laughlen Test fulfils this requirement in that it is completely reliable for the detection of syphilis in the untreated case, it may be carried out within ten minutes or less and it possesses a simplicity which may be claimed as unequalled. Further, this version of the reaction has been standardised in such a way that the reagent may be reproduced in an exact (scientific) manner. The large-scale testing for which the test was originally intended, therefore, seems to present the ideal field for this modified Laughlen Test: the numerous specimens from routine investigations, blood banks, ante-natal clinics, etc., can be tested easily and rapidly with the assurance that no positive serum will be missed, or at least none which would react positively with the Wassermann Reaction. As pointed out previously, this speed and reliability enable the test to be applied in several ways: it may be used as the preliminary screen test in a large serological centre in order to reduce the number of sera to be tested by the Wassermann Reaction; it is eminently suited for use as the standard diagnostic test in a "branch" laboratory: it is invaluable as a means of carrying out a test for syphilis at short notice since there is no need for any special preparation, the reagent being ready for immediate use from the moment it is 36 hours old until several months from the time of manufacture.

In Section Two it has been shown that this modified Laughlen Test possesses another very useful and interesting feature, i.e., it may be used as a most convenient method of carrying out research. In this way it has been demonstrated that the soya bean contains a substance similar to and reacting in the same way as syphilis "antigen" and although this substance presents problems of extraction, the fact that the soya bean is now cultivated on a large scale in many parts of the world gives weight to the suggestion that in this connection the plant is worthy of further research as it appears probable that in the soya bean there may be found substances which could be used with advantage as antigens in serological tests for syphilis; that these substances exist is certain, as has been proved in the present investigation, but there remains the problem of establishing standardised methods of extraction to produce antigen of high potency.

The investigation of sphingomyelin was of a more academic nature, being an attempt to probe the innermost structure of syphilis antigen, and here again the modified Laughlen Test provided a convenient method of conducting the enquiry. Even the somewhat negative results of the investigation do not detract from the preliminary argument that the known solubilities and insolubilities of sphingomyelin almost certainly establish it as a constituent of syphilis antigen and this argument is further strengthened by the results of the work on the soya bean where, it will be remembered, the best results were obtained with extracts made from ether-insoluble material. The purity of the sphingomyelin employed in the investigation leaves little doubt that the influence of this phosphatide on the activity of syphilis antigen is represented by the present findings i.e., that it is concerned with sensitivity but not with specific reactivity. It is not proposed to attempt an explanation of this property but the absence of glycerol in the sphingomyelin molecule might suggest the implication of the substituted base, sphingosine.

In conclusion, the reports of the three investigations which form this thesis may be summed up thus:- By an examination of the several components which constitute its reagent, the Laughlen Test for syphilis has been modified in such a way that it represents a standard, rapid and reliable exclusion test for the diagnosis of that disease. In addition, it has been shown how this version of the reaction was used as a simple method of carrying out original research: this revealed a potentially significant source of syphilis antigen in the soya bean and indicated that the sensitivity of syphilis antigens in general may be to some extent under the control of the phosphatide sphingomyelin.

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