CARDIOLIPIN ANTIGEN*

II. A QUANTITATIVE EXAMINATION OF SENSITIVITY

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In an earlier publication (Schmidt, 1951) "crude antigens" were compared with cardiolipin as to their sensitivity and specificity. The tests using the "crude antigens" were the Wassermann Complement-Fixation Reaction in Mørch's modification (WR-M), the Kahn Standard Reaction (KR), and the Meinicke Klärung II (MR). Cardiolipin was used in the Mørch modification of the Wassermann reaction (C-WR-M) and in a microflocculation reaction devised by the Venereal Disease Research Laboratory (VDRL). The investigation covered 1,322 sera selected by WR-M and KR. All samples that were positive in the screen test with WR-M and/or KR were, simultaneously with the quantitative evaluation in WR-M, examined qualitatively by C-WR-M, VDRL, and MR.

The experiments revealed that WR-M was significantly more often positive than C-WR-M in cases of "primary" and "fresh latent" syphilis ($\bar{\leq}$ one year since the infection), whereas no differences in sensitivity between WR-M and C-WR-M could be ascertained in cases of "secondary" and "older" syphilis. "Tertiary" syphilis was not classified separately, such cases as occurred being included with the "old" cases.

WR-M gave the greatest number of non-specific reactions, being significantly more non-specific than C-WR-M.

The purpose of the present investigation was to examine the *quantitative* relations between WR-M, C-WR-M, and KR at different stages of syphilis. The sera used were all submitted to parallel examinations in the three reactions, first qualitatively (one-tube examination), and then quantitatively (titration of serum); thus one reaction was not favoured more than another.

The *qualitative* evaluation of the reagin content ascertained whether the serum contained reagin, e.g. by ascertaining an existing inhibition of haemolysis in the complement-fixation tests or a precipitation in the flocculation reactions in one concentration of serum with a single antigen dilution. The reaction is termed positive or negative.

The *quantitative* examination ascertained the reagin content in a series of serum dilutions with a single concentration of antigen. Two reactions which are both positive at the qualitative examination may thus contain greatly varying amounts of reagin, which can be determined only by quantitative examination.

Material and Methods

The material comprised 3,980 sera, submitted to parallel tests with WR-M, C-WR-M, and KR. The sera were sent to the Sero-diagnostic Department by physicians and hospital wards, and the examinations undertaken were part of the department's routine work. The sera were not chosen according to previously fixed criteria, but on each of 20 separate days of examination about 200 sera picked out at random were tested. No corrections were undertaken for double examinations, which are estimated not to exceed 1 per cent. of the material examined.

After the examinations had been made, supplementary data concerning the patients in question were obtained. Besides the data accompanying the blood samples, each case was investigated by means of the Central Register of Syphilitic Patients (Jersild, 1919; Madsen and Krag, 1937; Schmidt, 1951). When samples could not be classified by the above methods, the authorities submitting them were asked for clinical and anamnestic information. The material was thus divided into two groups:

- (a) Patients with clinically and/or anamnesticly diagnosed syphilis, regardless of the time of infection.
- (b) Patients without syphilis.

The criterion for (b) was:

- (i) negative blood samples and absence of information about previous or present syphilis.
- (ii) sero-positive blood but suspicion of syphilis excluded, either because the patient's serum later became spontaneously negative, or because the patient had suffered from a disease with a known tendency to produce non-specific positive sero-reactions.

Only sera from syphilitic patients are discussed in this paper. All sera could be grouped as either syphilitic or non-syphilitic. There is no undetermined group, since the time of observation has been sufficiently long, viz. about 2 years.

No distinction was made between treated and untreated

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cases since the sera originated from many different hospital wards and physicians, and it would have been very difficult to obtain particulars of treatment.

Our attention has been drawn to the importance of the relation of the sero-reactions to the age of infection. This will be examined in a later series of investigations.

Technique

The technique is described in a previous paper (Schmidt, 1951). C-WR-M has proved to require somewhat more complement than WR-M in order to obtain the same degree of haemolysis. The fixation times differ for WR-M and C-WR-M, for which reason we shall give them once more, *viz.* :

WR-M : 45 minutes at room temperature plus 45 minutes at 37° C. before sensitized blood cells are added.

C-WR-M : 2 hours at 4° plus 30 minutes at 37° C. before the blood cells are added.

The haemolysis time is one hour at 37° C. for both WR-M and C-WR-M.

The initial serum concentration also differs in the three reactions performed :

In WR-M and C-WR-M it is 1 : 13 before the addition of the blood-cells.

In KR it is 10 : 11 before the addition of saline.

In the qualitative evaluation only reaction or non-reaction has been considered, *i.e.* plus or minus, whereas in the quantitative examination the potency of the serum has been determined in units (Kristensen, 1930 ; Schmidt, 1951). The unit of potency* chosen is $\frac{1}{2} \times \log 3$.

Present Investigations

The sera are divided into three categories :

(i) *Primary syphilis* (SI), characterized by typical chancre and/or *Spirochaeta pallida*.

(ii) *Tertiary syphilis* (SIII), characterized by the presence of cerebrospinal, cardiovascular, and specific osseous changes, or gummata.

(iii) "Rest", which comprises sera from patients with *secondary syphilis*, as well as that from patients with *fresh latent* and *latent syphilis*.

As there were only fourteen cases each of SI and SIII in the original material, the material was increased by examining over a certain period of time all sera received with definite information about primary or tertiary syphilis. These examinations were undertaken as parallel tests in WR-M, C-WR-M, and KR. The material supplementing SI and SIII may be considered to have been chosen at random.

* The term "potency" denotes that a standard is used for comparison which is the case for WR-M and C-WR-M. For KR the term ought to be "apparent potency" because no standard is used. This is because sera do not give the same degree of flocculation from one day to another even if the sera are kept frozen. A standard is not of the same importance for KR where only antigen and serum enter into the reaction, whereas in the complement-fixation reactions such variable factors as complement and blood cells have to be reckoned with.

In this paper the term "potency" will be used for all three tests.

The number of examinations in the different groups were :

SI : 14 + 82

SIII : 14 + 60.

"Rest" : 327 sera.

Out of 3,980 sera examined as a routine, 355 (*i.e.* 14 + 14 + 327) thus originated from patients with clinical and/or anamnestic syphilis (about 9 per cent. of the blood samples sent in). 24 sera (about 0.7 per cent.) were non-specifically positive in one or more reactions. This group will be specially accounted for in a later publication.

Qualitative Evaluation.—After the division into clinical groups, "2 × 2 tables" were made for each possible combination of the three reactions in the three clinical groups, nine altogether. Reactions which at the quantitative evaluation proved "non-readable" have not been included in the computation of the qualitative sensitivity, since they cannot enter into the quantitative determination of sensitivity. After the arrangement in "2 × 2 tables" the question arises whether or not the difference between the numbers of -/+ and +/- reactions is more than random. If theoretically they are equally distributed, the probability will be one-half for both combinations +/- and -/+, and the distribution over the two groups must conform to that case of the binomial theorem in which the probability of n reactions with a in one group, and $n-a$ in the other, is $\binom{n}{a} \left(\frac{1}{2}\right)^n$, and in which the

mean value is $\frac{n}{2}$. The observed deviation from this

value is $a - \frac{n}{2}$. From the binomial theorem we calculate the probability (P) of getting a deviation that is numerically greater than or equal to the latter. If P is less than or equal to 5 per cent., the hypothesis of equal distribution must be rejected, and one combination is said to occur significantly more frequently than the other. The values of P calculated for the present material may be seen in the Table opposite.

As previously demonstrated (Lundbäck, 1949 ; Vogelsang, 1950 ; Schmidt, 1951) WR-M is more frequently found positive in SI than C-WR-M. In respect of SIII and the "Rest" it has not been possible to show any difference in the incidence of positive reactions for the two complement-fixation tests. In previous papers (Schmidt, 1951 ; Reyn and Schmidt, 1951) WR-M was found significantly more often positive than C-WR-M in cases of "fresh latent" syphilis, but no difference

TABLE I
VALUES OF *P* FOR EACH POSSIBLE COMBINATION OF TESTS FOR SI, SIII, AND "REST".*

Serum Group	Result	Test		
		WR-M/C-WR-M	WR-M/KR	C-WR-M/KR
SI	Observed number +/—	43	25	4
	Observed number —/+	0	0	22
	Value of <i>P</i>	2.3×10^{-13}	3×10^{-8}	0.05 per cent.
SIII	Observed number +/—	5	7	10
	Observed number —/+	12	18	14
	Value of <i>P</i>	14.3 per cent.	4.3 per cent.	54 per cent.
Remainder	Observed number +/—	9	8	5
	Observed number —/+	6	24	24
	Value of <i>P</i>	61 per cent.	0.7 per cent.	0.05 per cent.

* "2 × 2" tables formed the basis for estimating values of *P*.

was found between WR-M and C-WR-M for secondary syphilis and "old cases", which comprised both latent and tertiary syphilis. In this paper the group "fresh latent" enters as a part of the "Rest", and not in a group of its own, the number of "fresh latent" cases in the material examined being too few.

In the present paper KR has in addition been compared with both WR-M and C-WR-M. For SI, WR-M is found to be significantly more often positive than KR, which in its turn is significantly more often positive than C-WR-M. In SIII, KR is just significantly more often positive than WR-M, whereas no difference could be found between C-WR-M and KR. Finally, KR is significantly more often positive than both WR-M and C-WR-M for the "Rest".

The following conclusions may be drawn from the qualitative estimation*:

SI.. WR-M > KR > C-WR-M

SIII.. KR > WR-M; KR = C-WR-M; WR-M = C-WR-M

"Rest".. KR > WR-M = C-WR-M

The differences in the incidence of positive reactions established by the qualitative estimation may be due to two causes,

(i) that the mean potency of one reaction is higher than that of the other, *i.e.* one reaction is more sensitive than the other;

(ii) that the standard deviation for one reaction is less than that for the other.

In fact, if two different reactions have an equal "mean potency" but different "standard deviations", the probability of 0-reactions is greater for that reaction with the larger "standard deviation". It can be decided only by quantitative examination to which of these causes the difference between two reactions is due. In an earlier paper (Schmidt, 1951) the difference in the number of positive reactions was interpreted as expressing the greater sensitivity of one reaction, and this may be based

on the fact that in smaller bodies of data examined quantitatively by both WR-M and C-WR-M, it could be seen that there was a quantitative superiority in favour of WR-M.

Quantitative Estimation.—When comparing the three sero-reactions, we are faced with the difficulty that the measurements do not give results below zero,* which means that there will be an accumulation of zero-values, as appears from Fig. 1, which shows the relations of the two complement-fixation tests for Group *iii*, the "Rest".

FIG. 1.—Relation between degrees of potency of WR-M and C-WR-M for sera from group "Rest".

Abscissa: C-WR-M degrees of potency.

Ordinate: WR-M degrees of potency.

If the distribution of sera according to their reagin contents is considered, they may be imagined to be divided into three groups:

- (1) Sera giving a degree of potency higher than zero.
- (2) Sera that are too weak to give positive reactions, but which might give positive reactions, *e.g.* in a higher concentration.
- (3) Sera which, regardless of all imaginable manipulations can never become positive.

* The reason why we may not have measurements below 0 is this: If a serum used in the maximal concentration prescribed for the technique yields more than 60 per cent. haemolysis, the only way to have haemolysis of 60 per cent. or below is to concentrate the serum by using larger amounts. In maximal concentration the dilution is recorded as 1 and its logarithm as zero.

* The signs denote: > significantly greater than
= not significantly different from.

Many of the patients may be expected to have been cured of syphilis by treatment. Provided that the incidence of biologically false positive reactions may be neglected, the cured patients ought to be "genuinely negative". Sera from these patients are not suitable for estimation of the relative sensitivity of the different tests.

From other investigations (Schmidt and Reyn, 1952) it is known that biologically false positive reactions as a whole are rather rare in the population (about 0.7 per cent. at the most). Since the present material may be accepted as a random sample in this respect only very few of such reactions should be expected.

In advance we do not know which patients are completely cured, but patients with at least one positive reaction in their sera can be regarded as true positive reactors. Again it is no proof of being "genuinely negative" that none of the three tests has yielded positive results. But if for example, for comparison of WR-M and C-WR-M we take only sera positive in KR into consideration we have certainly eliminated the "genuinely negative" sera. These conditional distributions have been analysed by the probit method. As stated in the statistical appendix it was found that they could be regarded as normal distributions though in some cases truncated at zero.

On this assumption "mean-value estimates" and "variance estimates" of potency have been

calculated for these conditional distributions. These "mean-value estimates" are denoted by the symbols $\overline{\text{WR-M}}$, $\overline{\text{C-WR-M}}$, and $\overline{\text{KR}}$ respectively. (See Appendix Tables IA and IB.)

As the standard deviations of these conditional distributions may be considered constant for the entire range of potency, only the pooled estimate of the variances for each set of calculations are stated in Appendix Table IB.

For the "Rest" the estimates of the variances lie about 3, whereas for SI and SIII they are about 6. This means that the differences in the degree of potency for the two reactions vary from one serum to the next with standard deviations of about 1.8 for the "Rest", and 2.5 for SI and SIII.

The "mean-value estimates" are plotted in Figs 2-10. For the comparison C-WR-M/WR-M the "mean-value estimates" calculated for a given KR for the two complement-fixation tests are designated $\overline{\text{WR-M}}/\text{KR}$ and $\overline{\text{C-WR-M}}/\text{KR}$, abscissa and ordinate respectively.

In the statistical analysis, it has been examined whether the relation between WR-M, C-WR-M, and KR may be described as a difference in the degree of potency which is constant apart from random deviations, for the entire range. If this were the case, the points in the diagram should arrange themselves about straight lines parallel to the identity line drawn into the Figure.

FIG. 2.—"Mean-value-estimates" for C-WR-M and KR for given positive value of WR-M for group "Rest".
Abscissa: "Mean-value-estimate" of C-WR-M.
Ordinate: "Mean-value-estimate" of KR.

FIG. 3.—"Mean-value-estimates" for KR and WR-M for given positive value of C-WR-M for group "Rest".
Abscissa: "Mean-value-estimate" of KR.
Ordinate: "Mean-value-estimate" of WR-M.

FIG. 4.—“Mean-value-estimates” for C-WR-M and WR-M for given positive value of KR for group “Rest”.
Abscissa: “Mean-value-estimate” of C-WR-M.
Ordinate: “Mean-value-estimate” of WR-M.

The “Rest”.—In the three diagrams (Figs 2, 3, 4) the points are scattered about the identity line. As far as this group is concerned, there are no significant differences between the three reactions examined. This must presumably be explained by the fact that the antigens have been mutually adjusted as to

sensitivity by the sera that most often occur in Denmark, i.e. sera from patients with latent syphilis, who make up by far the greater part of this group.

WR-M—C-WR-M

SI

All values are above the identity line (Fig. 5), signifying greater reaction with WR-M than with C-WR-M. There seems to be no constant difference in units of potency, as the points in the Figure are grouped about a straight line with a slope less than 1. It has been found that the difference between WR-M and C-WR-M is five to six units on the lower potency level and only about two units on the higher level. As the group SI comprises all patients with chancre and/or *Spirochaeta pallida*, regardless of the duration of infection, a part of the difference between the two complement-fixation tests may be due to the possibility that WR-M becomes positive sooner than C-WR-M. Even when both reactions have become positive, a clear difference in the unit of potency is maintained. Jersild (1936) showed that in SI WR-M becomes positive sooner than KR, which only becomes positive during the following week. At what point of time C-WR-M becomes positive in relation to the reactions carried out with the crude antigen, will be investigated later.

SIII

For this group there are only very few observations with degrees of potency above 5 (Fig. 6). As far as the lower degrees of potency are concerned no difference can be demonstrated between C-WR-M and WR-M.

FIG. 5.—“Mean-value-estimates” for C-WR-M and WR-M for given positive value of KR for group SI.
Abscissa: “Mean-value-estimate” of C-WR-M.
Ordinate: “Mean-value-estimate” of WR-M.

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FIG. 6.—“Mean-value-estimates” for C-WR-M and WR-M for given positive value of KR for group SIII.
Abscissa: “Mean-value-estimate” of C-WR-M.
Ordinate: “Mean-value-estimate” of WR-M.

FIG. 7.—“Mean-value-estimates” for C-WR-M and KR for given positive value of WR-M for group SI.
Abscissa: “Mean-value-estimate” of C-WR-M.
Ordinate: “Mean-value-estimate” of KR.

FIG. 8.—“Mean-value-estimates” for C-WR-M and KR for given positive value of WR-M for group SIII.
Abscissa: “Mean-value-estimate” of C-WR-M.
Ordinate: “Mean-value-estimate” of KR.

FIG. 9.—“Mean-value-estimates” for KR and WR-M for given positive value of C-WR-M for group SI.
Abscissa: “Mean-value-estimate” of KR.
Ordinate: “Mean-value-estimate” of WR-M.

FIG. 10.—“Mean-value-estimates” for KR and WR-M for given positive value of C-WR-M for group SIII.
Abscissa: “Mean-value-estimate” of KR.
Ordinate: “Mean-value-estimate” of WR-M.

$\overline{\text{KR}} - \overline{\text{C-WR-M}}$

SI

KR reacts somewhat more strongly than C-WR-M (Fig. 7), and most points in the Figure are scattered above the identity line, offset in favour of KR; the difference between units of potency in KR and C-WR-M may be considered constant. The average value (0.7 units) does not, however, differ significantly from zero.

SIII

C-WR-M reacts significantly more strongly than KR (average by two to three units), and the difference may be accepted as constant within the region examined (Fig. 8). Thus it is found, that the relations of WR-M and of KR to C-WR-M are the same for SI as for SIII, so that the two crude antigens react more strongly than C-WR-M for SI, whereas C-WR-M reacts as strongly as WR-M and significantly more strongly than KR for SIII.

 $\overline{\text{WR-M}} - \overline{\text{KR}}$

SI

WR-M is significantly more strongly reactive than KR (Fig. 9), all points in the Figure being situated above the identity line in favour of WR-M. The difference between KR and WR-M in SI may be regarded as constant. Whether this difference in the reaction of the antigens is due to the occurrence of different reagins in the serum at different points of time, or to differing degrees of sensitivity of the antigens to the same reagin, cannot be decided.

SIII

The difference between the two reactions cannot be considered constant for this group (Fig. 10). For the lower values WR-M is a little below KR, whereas the reverse is the case for the higher values.

The following conclusions may be drawn from the quantitative estimation :

SI.. $\text{WR-M} > \text{C-WR-M} = \text{KR}$
 SIII.. $\text{WR-M} = \text{C-WR-M} > \text{KR}$
 "Rest".. $\text{KR} = \text{C-WR-M} = \text{WR-M}$

Qualitative versus Quantitative Examination.—Qualitative examination discloses whether there is a difference between the reactions in the *number* of positive and negative values obtained. Quantitative examination discloses any differences in the *unit of potency*.

Differences disclosed by the qualitative examination must in principle be found again by the quantitative examination, either as a difference in the standard deviation, or as a difference in potency level. As the quantitative examination makes better use of the material under observation, it may demonstrate differences that could not otherwise be discovered.

Qualitatively WR-M was shown to be significantly more often positive than both KR and C-WR-M for SI. Quantitatively WR-M has also been shown to be more strongly reactive than either C-WR-M or KR for SI.

Qualitatively KR was found to be just significantly stronger than WR-M for SIII. Quantitatively constant conditions were not found for this group, WR-M being somewhat weaker at the lower values than KR, but the reverse being the case on higher potency levels. This means that KR will frequently be weakly positive where WR-M is negative. If both reactions are positive at a higher potency level, WR-M will on the average react more strongly than KR.

Krag (1938) reported similar observations, KR showing considerable preponderance over WR-M for degrees of strength 1–3, whereas for degrees of strength 4–9 WR-M occurred more often than KR. He concluded that a simple count of positive reactions was misleading, WR-M being generally more sensitive than KR, but KR has one zone (degrees of strength 1–3) in which it is superior to WR-M.

KR and C-WR-M did not differ for SIII qualitatively, but quantitatively C-WR-M was significantly more strongly reactive than KR.

Finally, KR was significantly more frequently positive qualitatively than both WR-M and C-WR-M in group *iii*, the "Rest", but by means of the quantitative tests used, no significant differences between the three reactions could be demonstrated. This is because the tests used are "overall" tests, whereas the entire qualitative difference is due to those sera which belong to the groups :

$\text{KR} = 1, \text{WR-M} = 0.$

$\text{KR} = 1, \text{C-WR-M} = 0.$

Possibly the accumulation of sera of $\text{KR} = 1$ may be attributed to the fact that the initial serum-concentration of KR is greater than that of WR-M or C-WR-M (see Technique).

Thus KR reacts more frequently than the complement-fixation tests with sera from patients with latent syphilis, but where all the three tests are positive, there is no difference in potency level.

Discussion

The purpose of this work has been to investigate and compare the quantitative sensitivity for WR-M, C-WR-M, and KR respectively. Qualitatively it had been shown that WR-M was significantly more sensitive than C-WR-M in SI and "fresh latent" syphilis, whereas no difference was demonstrated for the other stages of the disease.

It was previously found that in SII there were

no significant differences in the number of positive reactions between WR-M and C-WR-M.

On the presumption that, by means of another clinical classification of the material, it might be possible to demonstrate more differences between the different antigens, the material was divided into two clinically well-defined groups, (a) primary, and (b) tertiary syphilis and the "Rest", attention being paid only to the clinical diagnosis and not to the duration of infection or treatment.

Where sera from a miscellaneous group of patients are dealt with, it must be realized that the experimental conditions vary, *e.g.* the reagin content will probably be subject to both qualitative and quantitative variations from one individual to the next. By selecting sera from two well-defined clinical groups, it was nevertheless thought permissible to consider each group as a unit. As the sera examined are the same, and the reagin content at the time of examination must be constant for the individual serum, the differences observed between the mean values express the different effect of the antigens on the same reagin. This being the case, two courses are possible:

(1) To compare the potency of the different antigens towards a well-defined population of syphilitic sera.

(2) On the basis of a possible change in behaviour of the same antigen from one clinical group to the other, to demonstrate a change in quality in the reagin content.

The main difference between the two complement-fixation tests is the alcoholic antigen itself, the complement and the haemolytic system being the same, and the technique not differing in any essential points, apart from the fixation time. In WR-M the "crude" Mørch-antigen is used, and in C-WR-M the chemically better defined cardiolipin in conjunction with lecithin and cholesterol. Apart from some unknown lipoids and impurities in the WR-M antigen, these two antigens contain roughly the same components though in different individual proportions, and on the presumption that syphilitic reagin was unchangeably the same, *i.e.* identical in patients with SI and SIII, the relation between WR-M and C-WR-M should be expected to be the same in primary and tertiary syphilis. The relation between WR-M and C-WR-M might be imagined to be a constant (*i.e.* parallelism to the identity line), and it should thus be possible by an alteration of one antigen (*e.g.* by dilution, concentration, change of cholesterol content, etc.) to adjust the two complement-fixation tests to the same potency level.

It is found, however, that the mean difference in degree of potency between WR-M and C-WR-M

in SI and SIII is not the same. In SI the difference in "mean-value estimate" between WR-M and C-WR-M is significant, WR-M being more sensitive than C-WR-M. In SIII no significant difference can be demonstrated between the two complement-fixation tests. The fact that the relation between C-WR-M and WR-M is not the same in SI and SIII may indicate that the quality of the reagin changes during the progression of the syphilitic process. If the contention is correct that specific positive sero-reaction occurs only where there are living spirochaetes in the organism it might be possible that the spirochaetes cause different reagins to form at the different stages of the disease. From the pathological anatomy it is known how at different stages of syphilis spirochaetes selectively attack certain organs, and cause a decay of the part attacked. Consequently, it is probable that some substance in the spirochaete such as a hapten combines with a waste product from the decayed organ, and together with it stimulates the formation of reagin. Weil and Braun (1909) advanced the hypothesis that the syphilitic antibodies are caused by lipid antigen that is released from the tissues by the action of the spirochaetes. Considering the localization of the spirochaetes in the regional lymphatic node during the primary stage and in vessels and/or nervous tissue and skin in the tertiary stage, it might indeed be imagined that the products of the decay of these different organs might cause the formation of reagins of varying character.

It is well known that for most antigen-antibody reactions there is an area where antigen and antibody have maximum reaction, *cf. e.g.* the precipitation experiments with egg-albumin and anti-egg-albumin of Heidelberger and Kendall (1935). In the zone of equivalence there is neither free antigen nor free antibody; similar facts apply to most syphilitic sero-reactions, especially to the flocculation and precipitation reactions, *viz.* the so-called zone phenomenon. Heidelberger, Treffers, and Mayer (1940) showed that the optimum proportion between antigen and antibody in the precipitation reactions varies in different sera made by immunization with the same antigen. Differences in "combining ratio" in the precipitation reactions were particularly pronounced where antisera were produced in the same animal after short or long immunization periods.

Wallace, Osler, and Mayer (1950) found that the same facts apply to the complement-fixation tests, the complement-fixation ability per weight unit of antibodies varying with the length of the immunization period. Consequently the complement-fixation titre of an antiserum cannot be regarded as a direct measure of its content of antibodies.

The different reaction of the antigens may also be due to a difference in avidity, *i.e.* the rate at which sera combine with the antigen in question (Jerne, 1951).

Most syphilis antigens are adjusted (by admixture or dilution) so that maximum positive reaction is attained within rather wide limits with sera with varying reagin contents.

Thus for the adjustment of cardiolipin in complement-fixation tests, Maltaner and Maltaner (1945) used "reacting" sera, *i.e.* sera that fixed complement in a reaction with cholesterinized tissue extract as an antigen. Distinction was made only between reacting and non-reacting sera, no attention being paid to the duration of infection, or to treatment, if any.

Brown (1947) adjusted her macro-precipitation test with cardiolipin on the results of an examination of 3,877 sera "taken from syphilitic persons undergoing treatment, for the most part early cases treated with penicillin. There were also some instances in which heavy metals and malarial therapy were used. The group includes relatively few cases of neurosyphilis". Brown furthermore compared the serological findings corresponding to a few cases of treated syphilis with the "New York State Quantitative Complement-Fixation Test". She found that the results of the two reactions were not subject to any mutual simple mathematical proportion, and the presence or absence of reagin in serum could be recognized by one reaction earlier than by the other. She found, however, that where the reagin content in a serum was observed over an extended period with both reactions, it would show uniform serological conditions for the two reactions. The most pronounced discrepancies in titre seemed to occur in the early stages of the infection.

Eagle and Hogan (1940) demonstrated two antibodies in syphilitic sera, one antilipoidal and one antitreponematous. They found that, by absorption of a syphilitic serum with excess of beef-heart antigen, all the "reagin" could be removed, whereas the reagin-free filtrate continued to give positive complement-fixation and positive agglutination with a spirochaetal suspension, palligen, in the same titre as before the absorption with beef-heart antigen.

D'Alessandro (1946) thought that the antitreponematous antibodies appeared before the antilipoidal one in primary syphilis, whereas the antitreponematous and the antilipoidal antibodies should be found parallel in secondary and tertiary syphilis.

Andujar, Anderson, and Mazurek (1948) found

great divergence in "old syphilitic cases" between crude antigens and cardiolipin; they did not group according to clinical data.

Lundbäck (1949) divided his material into primary and other stages of syphilis, and by a partly quantitative examination found significantly greater sensitivity of WR-M in primary cases, whereas cardiolipin was more strongly reactive at all other stages.

Vogelsang (1950) found agreement between cardiolipin and crude antigen in 93.6 per cent. of 4,651 sera from syphilitic patients. Of the rest, one-fifth reacted with cardiolipin and four-fifths with crude antigen. Cardiolipin failed more markedly in cases of untreated syphilis.

Schmidt (1951) found WR-M more frequently positive than C-WR-M in patients with primary syphilis, and the same was the case for "fresh latent" patients. The latter group comprised patients who had had primary or secondary syphilis diagnosed within the past year, and who after treatment were free from symptoms at the time of examination, and also patients with positive sero-reactions and anamnestic data about syphilitic infection within the past year. In material examined later, this difference in the number of positive reactions between WR-M and C-WR-M in "fresh latent" syphilis is confirmed (Schmidt and Reyn, 1952).

Denecke (1950) states that sera from syphilitic patients are found to be more frequently positive and more strongly reactive with cardiolipin than with antigens of syphilitic liver extract and extract of beef hearts. The relation between the stage of the disease and the titre of the serum shows that the highest titres are found in SIII.

Denecke, Möllhausen, and Boeck (1950) think that the reagins demonstrated by the Wassermann test form a part of the syphilitic antibody complex, so that a high reagin titre runs parallel to a high antibody content. They find the highest reagin titres in patients with tertiary skin eruptions and in those with congenital syphilis showing florid skin manifestations.

Conclusion

In this paper it has been shown that in primary syphilis WR-M reacts significantly more strongly than C-WR-M, whereas no difference in reactivity can be shown between them in tertiary syphilis and the "Rest" (patients with any form of syphilis except SI and SIII). In primary syphilis, WR-M is significantly more often positive than KR, which again is more often positive than C-WR-M. No quantitative difference can be shown between KR and C-WR-M in SI. In the "Rest", there are no differences in reactivity between the

three reactions examined. In SIII, C-WR-M reacts significantly more strongly than KR, whereas no difference can be shown between KR and WR-M.

In quantitative examinations, special attention was paid to the comparison between the two complement-fixation reactions. The essential point of difference is the composition of the antigen. Cardiolipin is chemically a well-defined substance, whereas the composition of the crude antigen is relatively unknown. Nevertheless, it contains, in addition to the same substances as the purified antigen, a number of unknown components of presumably lipoidal character.

The difference demonstrated between the modes of action of WR-M and C-WR-M in SI and SIII seems to indicate that the reagin demonstrated in SI is not the same as that demonstrated in SIII. If the quality of the reagin were in fact constant, the relation between reactions for the two complement-fixation tests should be identical.

We thus think we can establish as a fact that the reagin content is changed by the progression of the syphilitic process. Whether the reagin that appears in primary syphilis persists and is supplemented with other components in the course of the infection, as suggested amongst others by D'Alessandro, or whether the early appearing reagin is replaced by another, cannot be decided on the basis of the present investigations.

Summary

(1) The quantitative relations of syphilis at different stages between Wassermann's complement-fixation test as modified by Mørch (WR-M), a complement-fixation test after the same technique using cardiolipin (C-WR-M) and Kahn's standard test (KR) have been investigated.

(2) 3,980 blood-samples were examined by the three different tests. The sera were divided into two groups :

- (a) from patients with syphilis, clinical or anamnestic ;
- (b) from patients without syphilis.

(3) Sera from patients with syphilis were subdivided into three groups :

- (i) from patients with primary syphilis (SI),
- (ii) from patients with tertiary syphilis (SIII),
- (iii) from all other syphilitic patients ("Rest").

No distinction was made between treated and untreated cases of syphilis.

The results of the examination of sera from group (b) will be published later.

(4) A brief account is given of the technique of the complement-fixation tests. C-WR-M needs more complement than WR-M to obtain the same

degree of haemolysis. (For further details see Schmidt, 1951).

(5) *Qualitative Estimation*.—WR-M is significantly more often positive in SI than KR, which again is significantly more often positive than C-WR-M.

In the "Rest", KR is significantly more often positive than both WR-M and C-WR-M, whereas no difference in the number of positive reactions can be demonstrated between the two complement-fixation tests.

In SIII, KR is significantly more often positive than WR-M, whereas no difference can be demonstrated between KR and C-WR-M or between WR-M and C-WR-M.

(6) *Quantitative Estimation*.—No difference in potency between WR-M, C-WR-M, and KR can be demonstrated in the "Rest".

In SI, WR-M is significantly stronger reacting than C-WR-M, the difference between the two complement-fixation tests being greater on the lower than on the higher potency level.

There is no difference between C-WR-M and WR-M in SIII.

There is no significant difference between KR and C-WR-M in SI.

C-WR-M is significantly stronger reacting than KR in SIII.

Finally WR-M is significantly stronger reacting than KR in SI, whereas no difference can be demonstrated with certainty between KR and WR-M in SIII.

(7) The investigations are discussed and an attempt is made to explain the fact that the relations between the different reactions are not the same in SI as in other stages of syphilis.

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