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KINETICS OF THE TREPONEMA IMMOBILIZATION REACTION UNDER IMPROVED CONDITIONS*†

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The demonstration by Nelson and Mayer (1949) of an antibody in syphilitic sera that immobilizes virulent *Treponema pallidum* in the presence of complement *in vitro* led to the development (Nelson and Mayer, 1949; Nelson and Diesendruck, 1951) of a *T. pallidum* immobilization (TPI) test for use in the case of individuals who might repeatedly give a positive reaction to other tests for syphilis but in circumstances suggesting that they might not in fact have the infection. Thus, TPI tests have been used to detect these biologic false-positive cases. However, there is a danger that other reactions, such as the destruction of complement, may lead to false-negative results, and it is generally recognized that provision against being misled by such results must be made: *e.g.*, by making terminal tests for residual complement in the reaction mixtures. Other unwanted reactions, such as the clotting of test mixtures, may likewise interfere with the attainment of a decisive result. In view of such difficulties, wide variations in the constitution of reaction mixtures and in the conditions of maintenance have been introduced by various workers (Nelson and Mayer, 1949; Nelson and Diesendruck, 1951; Khan, Nelson, and Turner, 1951; Magnuson and Thompson, 1949; Thompson and Magnuson, 1951; Portnoy, Harris, and Olansky, 1953; Reyn and Nielsen, 1953; Wheeler, van Goor, and Curtis, 1954; Boak and Miller, 1954; Wilkinson, 1954; Nielsen, 1957). Both deliberate and inadvertent variations in test conditions have hindered comparison of the results, especially on a quantitative basis, since no adequate account of their influence has been available. Nielsen (1957) has made an extensive survey of work in this field and has given estimates of variance in TPI tests by different workers. He has also estimated the contribution to the variance by different parts of the techniques employed. The objectives of the present investigations have been:

(1) to remove some of the causes of disturbance;
(2) to study the kinetics of the reactions under improved conditions.

By the preliminary assay§ of sodium thioglycollate preparations just before use, and the rejection of all that showed less than 90 per cent. of theoretical potency, it has been found unnecessary to augment the amount used in the basal medium above that originally recommended by Nelson and Diesendruck (1951), though other workers have used up to five times as much. The careful maintenance of anaerobiosis during the extraction of treponemes and preparation of test suspensions and during subsequent reactions, wherever practicable, has avoided any need to use greater concentrations of thioglycollate.

Another variable component has been the testicular tissue juice extracted with treponemes that most workers have allowed to remain in suspensions used for reaction mixtures, considering it to contain some component essential to the satisfactory survival of treponemes in controls. Rice and Nelson (1951)

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§ Weigh a sample (0·2 to 0·3 g. to the nearest 0·001) and dissolve in 40 ml. redistilled water (preferably redistilled after addition of $KMnO_4$ and H_2SO_4); add 12 ml. of approximately normal HCl and boil for 2 minutes. Cool and titrate with 0·1 *N* iodine solution, adding about 1 drop of 1 per cent. starch solution as indicator toward the end.

$$\text{Per cent. } SHCH_2COONa \text{ by weight} = \frac{11{\cdot}41 \text{ (iodine normality) (titre in ml.)}}{\text{Sample Weight (g.)}}$$

The *iodine normality* is determined as usual by titration with standard sodium thiosulphate solution. The assay is essentially in accord with procedure given by U.S. National Institutes of Health (1946, 1947, 1955).

suggested that it might be replaced or reinforced by beef-serum ultrafiltrate or by a "treponema survival factor" (TPSF) that they isolated from beef serum; but the practice of including tissue juice in the treponemal suspensions has continued. Investigations by Portella (1954, 1955) indicated that it was even advantageous to remove tissue juice as completely as practicable, thus avoiding its anti-complementary and thromboplastic activities. Instead, a modified medium was provided that supported survival of 90 or more per cent. of treponemes in controls for periods of 48 to 60 hours. Thus an important disturbance by the destruction of complement or by the clotting of test mixtures was avoided, and satisfactory conditions were established with the aid of other technical modifications for a study of the kinetics of the immobilization reaction.

The present purpose is to report some of the results that show the relation between the amount (z) of test-serum used per ml. reaction mixture, the amount (x) of complement, the time (t) elapsed since its addition, and the resultant degree (y) of immobilization attained. This indicates how an account may be taken of these factors to aid expression of serum antibody titre, and affords a variety of conditions for its evaluation from which those most convenient for a given case may be chosen.

Materials and Methods

A specimen of the stock Nichols strain (Nichols and Hough, 1913) of *T. pallidum* in frozen testicle was obtained in 1954 from Dr. John F. Kent of the Army Medical School, Washington, D.C. The strain was maintained as usual by frequent serial transfers in rabbit testicles, the animal quarters being maintained at 16°C.

Preparation of Treponemal Suspensions.—Both testicles of a rabbit that had developed orchitis in 7 to 10 days after inoculation were used within 48 hours for treponema harvesting. This was done aseptically, as usual, but at 16° rather than 35° C. The sliced testicles were treated with 60 ml. of Medium I, which ordinarily was the same as Nelson's original medium (Nelson and Diesendruck, 1951). As mentioned previously, care was taken to ensure that thioglycollate stocks had at least 90 per cent. of their theoretical potency. The gaseous atmosphere over suspensions of treponemes during their extraction from testicular tissue in flasks and their later maintenance in tubes in Brewer anaerobic jars came from a carefully analysed gas stock, containing 5 per cent. carbon dioxide in nitrogen. An adaptation (Allen and Thompson, 1954) of the method of Van Slyke and Hanke (1932) was used to estimate the oxygen content of the stock, and for occasional checks upon the final gas contents of extraction flasks and Brewer jars. The oxygen content of gas stocks usually appeared to be about 0·013 per cent. or less; it was never greater than 0·03 per cent., otherwise it was rejected. To aid in establishing anaerobiosis, an air-tight evacuation-filling system was used. In an anaerobic extraction flask, the sliced tissue in Medium I was agitated by using a Kahn-type shaker for one hour in the room at 16°C. Then a preliminary centrifugation in the usual way yielded a debris-free treponemal suspension as supernate. Such suspensions of treponemes, still containing the simultaneously extracted tissue juices, have been used by other workers for TPI tests. However, we have separated the treponemes from nearly all the tissue juice by re-centrifuging the first supernate for 30 min. at about 8,000 g. (7,000 r.p.m. in a Servall SS-2 angle-type vacuum centrifuge).

The tubes, 12 ml. heavy-wall pyrex, 17·5 × 102 mm. with rubber adapters (Servall Nos. 102A and 302), were plugged with gauze surrounding an inverted rubber nipple, with a shoulder that served to prevent drawing the plug down into the tube during centrifugation. The second supernate was decanted and the residue was used, re-suspended in Medium II, which contained twice as much cysteine hydrochloride and glutathione and four times as much beef-serum ultrafiltrate as Medium I, but was otherwise the same. In such re-suspensions, the amount of medium was taken (120 ml. for the present experiments) so that about five to ten treponemes would be obtained in counts per microscopic field, with high-power non-immersion magnification about 400 times. Such suspensions were used for test mixtures, 0·8 ml. per ml. of final volume. Not only were the above-mentioned vitiating influences largely eliminated, but a suspension remarkably low in concentration of host antibody appears to have resulted. By inadvertence, in Experiments 1, 2, and 3 (reported below) only nine-tenths as much cysteine and glutathione were used.

Preparation of Complement.—The complement for this work was obtained by pooling clear sterile sera from about 25 guinea-pigs. The pool was dispensed in 1·5 ml. amounts in screw-capped tubes, "shell" frozen, and stored at −70°C. until needed. The haemolytic activity of the complement was determined by the technique of Wadsworth, Maltaner, and Maltaner (Wadsworth, 1947), using the haemolytic system with 15-minute incubation. The undiluted pooled guinea-pig serum usually contained one unit (50 per cent. effective amount) in about 0·0011 ml. Although complement dilutions were made and x expressed in terms of the 50 per cent.-effective haemolytic unit, it is well known that for different preparations fixability should not be considered as necessarily proportional to haemolytic activity. Sera from a fairly large number of guinea-pigs are pooled with a view to making the averaged activities approximately proportional.

Test Serum.—In the present experiments, the test serum was a sterile specimen obtained from a rabbit that had had untreated syphilitic infection for about 21 months. The dilutions were made in sterile 0·85 per cent.

sodium chloride solution with portions of the serum that had been inactivated by heating in a water bath at 56°C. for 30 minutes.

Test Mixtures.—All measurements in the dilution of serum and complement and in the constitution of test mixtures were made with pipettes that had been recalibrated so that their contribution to error should not exceed 1 per cent. According to the requirements for each test mixture to be made, a preliminary dilution (S) of test serum was made to contain $10z$ ml. serum per ml.; and likewise, a complement dilution (C) was made to contain $10x$ units per ml. These and subsequent mixtures were prepared in the indicated proportions in quantity sufficient for the experimental purposes. For the preparation of test mixtures 0·1 ml. serum dilution S was mixed with 0·8 ml. treponemal suspension and incubated at 35°C. for 12 hours. Then to aliquots of such mixtures 0·1 ml. complement dilution (C) was added per 0·9 ml. serum-treponeme mixture, and approximately 1 ml. aliquots of the resultant serum-treponeme-complement mixture were transferred to individual replicate tubes for further anaerobic incubation at 35°C. Accordingly, such mixtures contained z ml. test serum and x units complement per ml. In each experiment, duplicate or quadruplicate sets of tubes were used, each set having tubes respectively with z in the approximately geometric series, 0·01, 0·00316, 0·001, 0·000316, 0·0001, 0·0000316, and 0·00001, and the same value of x. A control for each set had $z = 0·01$ and the same amount of an inactivated portion of the complement preparation, inactivation having been effected by 30 minutes' maintenance in a water bath at 56°C. A complement or inactivated complement was added, as stated previously, after the preliminary incubation for 12 hours; t is used to denote the time in hours that elapsed thereafter. Anaerobic incubation at 35°C. was continued until just before readings of the degree (y) of immobilization were to be made.

Counts and Evaluation of y.—Reaction mixtures were gently agitated to ensure fairly uniform suspension of treponemes when samples were taken for reading. By dark-field microscopy, 100 treponemes were counted in the usual manner, and a note taken of the number (L) of these that were motile. A similar count (L_c) of motile treponemes in 100 examined in the corresponding control of the given set was made. The time (t) of reading was taken as midway between the start and finish of counts for the given set, approximated to the nearest 0·01 hour. As usual, the degree of specific immobilization $y = (L_c - L)/L_c$; this was computed or read from a table to the nearest thousandth. If subsequent readings were to be made, the reaction tubes were returned promptly after sampling so that they could be maintained at 35°C. anaerobically. All observations were made by the senior author.

Other Controls.—With each set there were also two other controls that contained beef-serum ultrafiltrate instead of test serum, one having x units active complement per ml., the other having a similar amount of the inactivated complement. Though not used in the computation, these controls provided the usual safeguards against vitiating influences that might otherwise be unsuspected.

Estimation of Median-Effective z.—Thus, for a given set, read at a given time (t), there was obtained a set of readings (y_k) corresponding to values (z_k) in the geometric progression of test-serum dilutions; but usually only enough tubes were read to ensure that some values of y_k would be less and some more than 0·5 for the given set. Then Z was estimated by interpolation as the value of z that should have yielded a value of $y = 0·5$; Z is called the median-effective dose (50 per cent.-effective dose) for the given time (t) of incubation with the given amount of complement (x) per ml. reaction mixture.

Approximately, it was found that, for given x and t, the logarithm of z was linearly related to the logarithm of $y/(1 - y)$; *i.e.* for given x and t,

$$\log z = \log Z + h \cdot \log [y/(1-y)] \quad \ldots . (1)$$

may be considered as a fair approximate relation for the purposes of graduation or interpolation to estimate the median-effective dose, Z. This may be done conveniently from a graph of the data plotted with log z versus logit y on Berkson's logistic graph paper (No. 32450, Codex Book Co., Norwood, Mass.); of course, Z is the value of z when $y = 0·5$. In the present work, simple linear interpolation was used on the graph between points with y-values straddling 0·5; alternatively, Berkson's tables of logits were used with linear interpolation for log Z (Berkson, 1953). The latter is more convenient when a calculating machine is available, and is extremely simple. However, it is not intended to suggest that Equation 1 holds except in the neighbourhood of $y = 0·5$ approximately, in order to permit its use in the estimation of Z.

The point of view from which such estimations are made in many situations and a comparison of other principles of estimation of median-effective dose have been presented elsewhere in a general review of bio-assay (Thompson, 1950a). Emphasis was laid upon the close agreement to be expected between estimates of median-effective dose made by the use of the logistic curve or of the integrated normal curve as in probit analysis. Attention was also directed to the case of assays based upon complement-fixation reactions where either median-effective dose for given time or median-effective time for given dose is to be estimated. It is apparent that the latter has an important bearing upon the present work.

Experimental Procedure

Experiment 1.—Duplicate sets were prepared with $x = 20$, 40, and 80. In each set the series of values of z from 0·01 to 0·00001 was used together with the usual controls. Readings were made with t = about 12, 24, 36, and 60 hours.

Our previous work had indicated that the median-effective dose (Z) would be approximately inversely proportional to the product (xt^2) of the complement concentration and the square of the elapsed time. The results of Experiment 1 are given in Fig. 1 with $10^4 \cdot z$ as ordinate and $10^5/xt^2$ as abscissa. Points are shown in circles for $x = 20$, in triangles for $x = 40$, and in squares for $x = 80$. Since the rectangle for the ordinate between 0 and 10 and the abscissa between 0 and 5 would be too closely packed with points, or the rest of the graph too greatly extended, a 10-fold magnification of the rectangle is shown in Fig. 1 to the right of the main graph. Points within the rectangle are not shown in the main graph. The straight line was fitted to all the points with a view to minimizing the sum of the squares of their vertical deviations from the line in accordance with well-known procedures. In the small-scale graph the line appears to pass almost through the origin, the ideal situation that would correspond to the absence of antibody from sources other than the test serum or to its presence in only a negligible amount. However, in the magnified portion there is some indication that a small amount of antibody may be present. In that region, the contribution of antibody from the test serum is small because z is small and, relatively, the amount of antibody from other sources may not be negligible. The fitted line in Fig. 1 may be given as:

$$Z = a + b/xt^2 \text{ with } a \simeq -6 \cdot 9(10^{-5}) \text{ and } b \simeq 28 \cdot 9 \ldots (2)^*$$

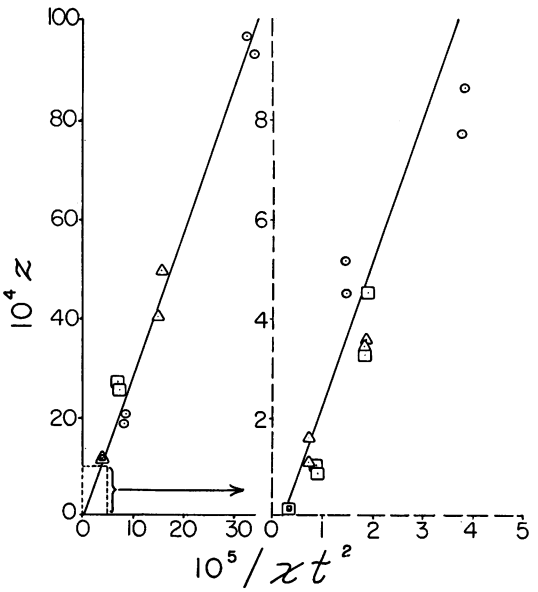

FIG. 1.—Points of Experiment 1 given in circles, triangles, or squares respectively indicate that the complement concentration (x) is 20, 40, or 80 units per ml., t = reaction time in hours, and Z is the concentration of test serum estimated by the method described in the text as required to yield 50 per cent. immobilization. A portion of the graph is shown on the right ×10. Observed points within this region are not indicated on the main graph.

* The sign $\simeq$ denotes "is approximately equal to".

Experiment 2.—This was like Experiment 1, but quadruplicate sets were used with $x=80$ throughout, and t was limited to values between 12 and 38 hours. The results are given similarly in Fig. 2, with the points in the squares indicating that $x=80$ in accordance with the previous scheme. Good agreement appears among the replicates—inadvertently, the reading of one of the quadruplicates was omitted for t about 24 hours. A cross on the graph shows the centroid in each case, the position having the mean ordinate and mean abscissa value of the replicates. A straight line, fitted to the eleven observed points as before, is shown in the graph; the crosses lie almost on it. In the form of Equation 2 the line here would have $a \simeq -2 \cdot 6(10^{-4})$ and $b \simeq 76 \cdot 9$. Again, there appears to be some suggestion of a small amount of antibody from sources other than test serum.

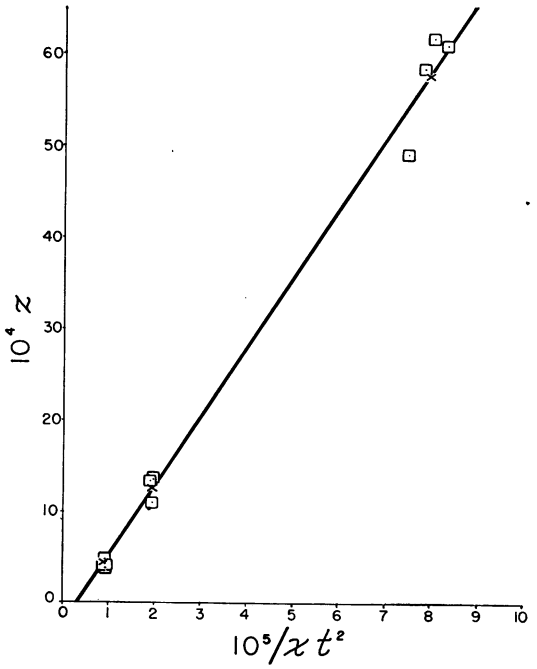

FIG. 2.—Points of Experiment 2 given in squares, indicating $x=80$. The centroids of replicate observations for about the same t are shown as crosses.

Experiment 3.—This used a portion of the treponemal suspension that had been prepared for Experiment 2 but had been set aside and maintained anaerobically at 35°C. for 2 hrs before use. The same test serum was used in the identical series of dilutions, but a different complement preparation was used. Duplicate sets of reaction mixtures were made with $x = 20$, 40, and 80, respectively. Readings were made with t between 12 and 38 hours. Results are given in Fig. 3 in the same manner as before with circles, triangles, and squares indicating $x = 20$, 40, and 80, respectively. The corresponding fitted line has $a \simeq 1 \cdot 2(10^{-4})$ and $b \simeq 43 \cdot 2$.

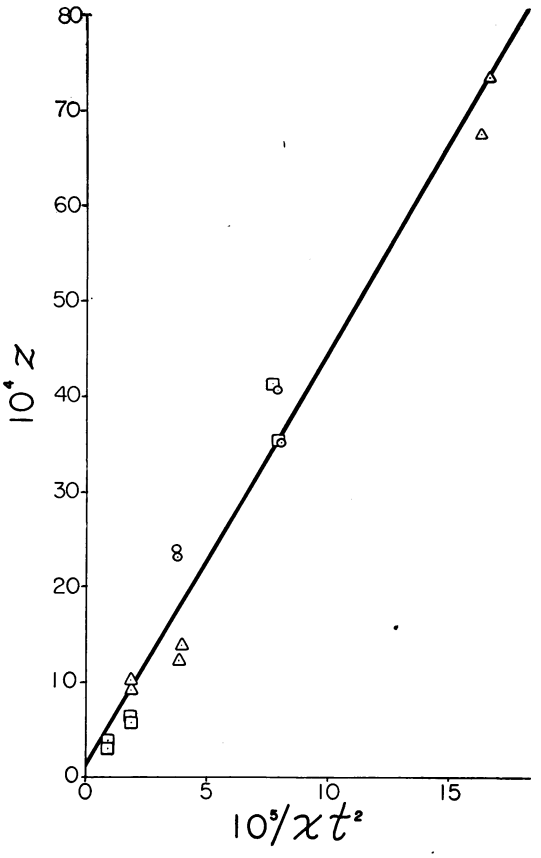

FIG. 3.—Points of Experiment 3 shown in accordance with the scheme of the preceding Figures.

Discussion

All data obtained in the experiments are shown in the graphs. The three experiments were run just before the work was interrupted for 6 months by the return of the senior author to his post in Brazil in April, 1956. In all the experiments, it is strongly indicated that little or no specific antibody is contributed to the reaction mixtures by any source other than the test serum. Indeed, any such contribution would appear to be negligible if t is taken between 12 and 26 hours. Then, within any given experimental run, the resultant degree (y) of immobilization appears primarily dependent upon the product of the amount (z) of test serum per ml., the amount (x) of complement, and the square (t^2) of the time elapsed since the addition of the complement. Obviously, different slopes (b) are exhibited in the lines respectively fitted to the results of the three experiments.

Here, comparisons are made for estimated combinations of z, x, and t that should yield 50 per cent. immobilization ($y = 0{\cdot}5$); and Z is estimated by interpolation as the value of z required for given values of x and t. It is readily seen that we could, instead, have estimated X as the value of x required for given values of z and t, or we could have estimated T as the value of t required for given values of z and x. This suggests that we might choose to use, instead of a series of values of z, a series of values of either x or t, or a suitable lattice of various values of these variables (z, x, t) and an interpolation or graduation formula of the form,

$$\log zxt^2 = \log K + h \cdot \log [y/(1-y)] \quad \ldots . \quad (3)$$

This we may suppose to hold approximately within the neighbourhood of $y = 0{\cdot}5$ to an extent great enough to permit a satisfactory estimation of K. Then, obviously, K is the estimated value of zxt^2 that should yield 50 per cent. immobilization.

In most experiences with biological dosage indicator systems, it appears that it is best (Bliss and Cattell, 1943; Thompson, 1950a) to test a reference-standard preparation simultaneously with the test of any unknown preparation, and to base the assay of the latter upon the ratio of their potencies as indicated in the given test run and the known (or defined) potency of the reference standard. This is usually distinctly preferable to the use of the biological indicator system as a standard, *i.e.*, the treponeme-complement system in the present case. Bliss and Cattell (1943) cite, as a unique exception to the rule in the field of bio-assay, the use (Bliss and Packard, 1941) of the eggs of *Drosophila melanogaster* both as an indicator of x-ray dosage and as a reproducible standard.

In accordance with the principle of comparison of test serum and reference-standard serum in the same experimental run with the same treponeme suspension and the same complement, the corresponding values for K obtained may be denoted simply by K and K_o, which should be approximately in inverse proportion to their respective potencies, p and p_o. Hence, we may estimate the test-serum potency by

$$p = p_o K_o / K \quad \ldots . \quad (4)$$

How much, if anything, this contributes to precision remains to be investigated. Nielsen (1957) has presented such a study, but under conditions very different from those proposed here. Relative titres of two sera may vary greatly, either with different complement or treponeme preparations; the latter has been pointed out by Nielsen.

Equation 3 resembles a combination of relations that were separately used by von Krogh (1916) and developed by Thompson (1950b) with a variable

power (r) of t; it was also used by Almeida (1950) with $r = 1$ for timed reactions to attain a stipulated degree (y) of haemolysis in the complement-fixation reaction with cardiolipin antigen and syphilitic serum. The principle has recently been used in titration of complement by Plescia, Amiraian, and Heidelberger (1956), who found in some cases that $r \neq 1$ was required*.

Now, let a pair of tests be considered with the same serum and other reagents run simultaneously with the same value of x, but with reaction times t_1 and t_2 respectively, about 12 and 24 to 26 hours. Let the estimates of Z be denoted correspondingly by Z_1 and Z_2, and let $K_1 = Z_1 x t_1^2$ and $K_2 = Z_2 x t_2^2$ be the corresponding estimates of K. Then, for such pairs of estimates, we may study the relative numerical discrepancy, defined as $R = \pm 2(K_1 - K_2)/(K_1 + K_2)$ but not negative. The three experiments above provided thirteen such pairs, and previous experiments provided sixteen other pairs, essentially in accordance with this plan; a total of $n = 29$ pairs and corresponding values of R were thus available. These are indicated by points in Fig. 4, having $100R_k$ as abscissa taken in order of increasing magnitude and having $\bar{p}_k = k/(n + 1)$ as ordinate, respectively. In accordance with principles of statistical inference developed elsewhere (Thompson, 1936), $\bar{p}_k$ is the probability that a random future pair of tests made under the stipulated conditions would yield a value of R less than that of R_k. Successive points are joined to form a graph as a guide to the eye and an aid to interpolation; the ordinate ($\bar{p}$) is called the cumulative probability. Accordingly, it appears that in such work we should expect relative discrepancies of less than 15 per cent. to be encountered for about half of the time.

Here, we use only the simplest features of a system of statistical inference based upon an assumption economy (Thompson, 1936, 1949) that involves little beyond what the experimenter must assume, if he aims to study an approximately reproducible phenomenon. The system was independently developed by Savur (1937) and it has been widely recognized (Wilks, 1948). Applications in various fields have appeared (Wadsworth and Hyman, 1938; Maltaner and Thompson, 1943; Thompson, 1952; Griffin and Thompson, 1956).

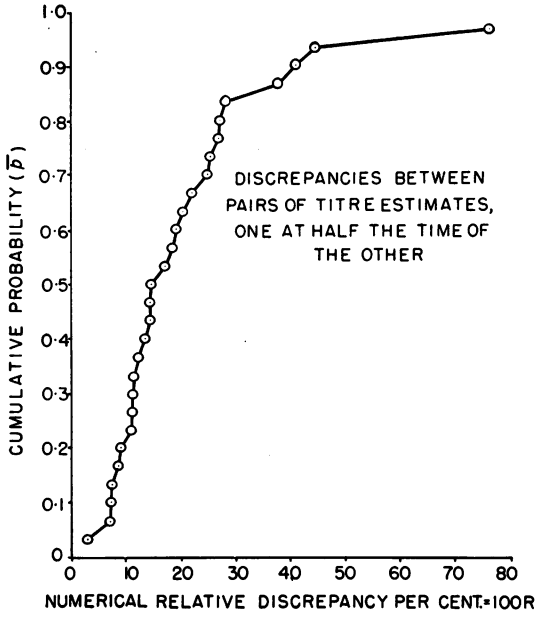

FIG. 4.—The points have values of $100R$ as abscissa in order of increasing magnitude *versus* $\bar{p}_k = k/(n+1)$ as ordinate, where $n=29$, the number of test-pairs. The ordinate estimates the probability that $100R$ for a future pair of tests under the stipulated conditions would have a value less than that of the corresponding abscissa.

Summary

The kinetics of the *T. pallidum* immobilization reaction have been studied under conditions aimed at lowering thromboplastic and anti-complementary activities of the treponemal suspensions by extraction from testicular tissue at lower temperature, separation from most of the tissue juice, and resuspension in a modified Nelson's medium. The survival of 90 per cent. or more in controls for 48 hours is attainable. The sensitivity varies proportionally with the concentration of complement and with the square of the reaction time. This permits advantageous use of protracted reactions with highly diluted sera that would otherwise be too toxic for treponemes or too anti-complementary, since doubling or tripling the reaction time respectively makes the system four or nine times as sensitive. Two relative titre estimates on the same serum with reaction times respectively about 12 and 24 hours differ by more than 15 per cent. only for about half of the time. For a comparison of the potencies where different reagent preparations are used, recourse may be had, as usual in bio-assay, to the use of a reference-standard serum run simultaneously with the same reagents in each case.

* The symbol $\neq$ denotes "not equal to".

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