# TUBERCULIN STUDIES
# RELATIVE TO POTENCY DETERMINATIONS

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## INTRODUCTION

Some indication of the important rôle occupied by tuberculin in Canadian veterinary medicine may be gathered from the amount supplied veterinary inspectors employed by the Dominion Department of Agriculture for the diagnosis and control of bovine tuberculosis throughout Canada, and which, according to the figures of the Chief Pathologist, as given in the 1933 Report of the Veterinary Director General, amounted to 2,104,220 test doses for the year ending March 31, 1933. This extensive and almost ubiquitous use of tuberculin in Canada makes it imperative that a standard product of adequate potency be in the hands of veterinarians who employ it in order to obtain uniformly the best results.

Until recent years the method generally employed for determining potency of tuberculin for use in veterinary medicine on the North American Continent was by the lethal dose method, which discounting modifications as practised in different laboratories is essentially the German official test, known generally as the Frankfurt or lethal dose method and is based on the fact that tuberculous guinea pigs will succumb to the inoculation of potent tuberculin.

At best, the determination of potency by this method is crude, for in the final analysis potency can only be indicated by the loose nomenclature, superpotent, potent, subpotent or worthless, in comparison with another tuberculin which had given satisfactory results, instead of by more definite and accurate terms of potency units per cc.

In an endeavour to correct this defect, Watson and Heath published in 1924 the results of their experiments for standardizing tuberculin by complement fixation methods, but so far their work has not been corroborated, and in fact, has been criticized and questioned as a reliable method of indicating tuberculin potency. Their conclusions were based on results obtained with tuberculins prepared from culture filtrates of tubercle bacilli grown on glycerinated bouillon, and in certain quarters it has been implied that their results might be due to the glycerinated bouillon content of the tuberculins tested and not to any specific tuberculin properties.

In order to challenge the possible speciousness of the criticisms advanced against the complement fixation method for standardizing tuberculin and in view of the probability that tuberculins prepared from filtrates of tubercle bacilli grown on synthetic media may eventually replace tuberculins prepared from glycerinated bouillon cultures filtrates, it appeared desirable to prepare tuberculins from filtrates of synthetic media in which representative strains of tubercle bacilli had been grown and to determine the activity of these products comparatively by complement fixation and *in vivo* methods

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The following experimental studies made over a period of seven years (1927-1933) concern comparative potency determinations by *in vivo* and *in vitro* methods of synthetic tuberculins prepared under different conditions and treated with a variety of reagents, along with related observations and evidence to explain paradoxical results that may be encountered in utilizing the complement fixation method for indicating potency.

## I. THE PRODUCTION OF TUBERCULIN IN SYNTHETIC MEDIA

Among formulæ for synthetic media recently recommended for the cultivation of tubercle bacilli are those of Long (1) and Dorset, Henley and Moskey (2). Preliminary comparative tests were made with these two media to determine which would better support the growth of tubercle bacilli. On this basis the results favoured the latter medium and it also had the added attraction of containing two less ingredients. An undetermined precipitate, however, often appears after sterilization, but disappears during growth of cultures planted on it.

Although this medium furnished luxuriant growths with certain strains, it, nevertheless, cannot be considered a universal medium for cultivating bovine strains of tubercle bacilli and in this respect cannot be compared with glycerinated bouillon, for in comparative tests of eleven bovine strains, all of which grew well on glycerinated bouillon, only three of them grew on this synthetic medium. On the other hand, eight avian and two human strains grew equally as well, and in some cases more profusely, on the synthetic medium as on the glycerinated bouillon.

Of the mammalian strains that did grow on synthetic medium four were selected for the production of tuberculin and comprised the well-known strains H37, B.C.G. and Vallée, and a bovine strain of low pathogenicity identified as No. 110. Three subcultures from each of these four strains were planted in three separate flasks, containing sterilized synthetic medium of the chosen formula, prepared at one time, after which all twelve flasks were incubated.

On the forty-fourth, sixty-sixth and one hundred and third days after first incubation, four flasks, each representing a subculture of a different strain, were removed from the incubator, heated at 90° C. for one hour and on the following day the bacillary growth from each flash separated on individual weighed filter papers. The growths were washed on their respective filter papers with distilled water and the filtered washings added to the corresponding culture filtrates in a quantity to bring them back to the original volume of media. After passage through an N Berkefeld filter, the acidity of each culture filtrate was determined by phenolphthalein, then concentrated over a water bath to one tenth the original volume of medium employed and 0·5 per cent phenol added. The temperature of the culture filtrates during concentration was never over 78° C. The weights of the bacillary growths were determined separately after drying in the incubator for ninety-six hours.

In the medium on which strain Vallée grew for sixty-six days, white opaque bodies, resembling bits of white thread about one-eighth of an inch in length were formed. These were found in the bottom of the flask and were insoluble in either water, alcohol, ether or glycerine. This was the only culture revealing this phenomenon. In two of the three flasks in which H37 was cultivated, for forty-four and sixty-six days respectively, white fine precipitates were formed. These were fine enough to pass through No. 2 Whatman filter papers. It was further noticed that the culture of H37 grown for sixty-six days did not utilize the iron in the medium as well as the other two cultures from this strain, or as well as the cultures from the other three strains and the filtrate when concentrated remained opalescent for more than a year, after which it cleared.

In table 1 is given condensed information regarding these twelve tuberculins with respect to their identity, age of cultivation, original volumes of medium employed and the volumes of filtrates recovered, with their reactions to phenolphthalein and the weight of bacillary growth recovered from each filtrate.

COMMENT

While realizing the defects of phenolphthalein as an indicator for determining true acidity, nevertheless, it will be apparent in a review of table I, that the culture filtrates of all four strains were less acid than the original media, and with the exception of B.C.G. which remained constant over the periods tested, the acidity of the others inclined to diminish with age.

Eleven of the twelve tuberculins after concentration produced precipitates of varying amounts, the exception being Tuberculin No. 47, prepared from the culture filtrate of strain No. 110 after growth for sixty-six days. As the culture filtrate representing this tuberculin was less acid than any of the others, as will be noted in table 1, it was considered probable that to this might be attributed the absence of a precipitate in the concentrated tuberculin. This hypothesis was indirectly supported experimentally for it was found that almost all the precipitates found in synthetic tuberculins after concentration could be re-dissolved by the addition of normal soda solution; and also, the addition of normal hydrochloric acid to clear solutions of tuberculin prepared in the usual manner produced precipitates which could be again made soluble by the addition of normal soda solution.

TABLE 1

| Identity of Tuberculin | Strain | Ages of bacillary growth (in days) | Synthetic Media | | Culture filtrates | | | Weight of recovered bacillary growths in grammes | Percentage weight of growth to | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Amount | Reaction to phenolphthalein | Amounts recovered | Percentage of medium lost | Reaction to phenolphthalein | | Original volumes of media | Recovered culture filtrates |
| | | | | % | | | % | | | |
| 40 | B.C.G. | 44 | 250c.c. | +3·5 | 165c.c. | 34·0 | +0·75 | 1·85 | 0·74 | 1·12 |
| 41 | " | 66 | 250c.c. | " | 150c.c. | 40·0 | " | 2·25 | 0·9 | 1·5 |
| 42 | " | 103 | 250c.c. | " | 120c.c. | 52·0 | " | 2·8 | 1·12 | 2·3 |
| 43 | Vallée | 44 | 350c.c. | " | 250c.c. | 28·5 | +1·15 | 2·12 | 0·605 | 0·84 |
| 44 | " | 66 | 350c.c. | " | 240c.c. | 31·4 | +1·25 | 1·5 | 0·428 | 0·62 |
| 45 | " | 103 | 250c.c. | " | 140c.c. | 44·0 | +0·75 | 2·4 | 0·96 | 1·7 |
| 46 | No. 110 | 44 | 350c.c. | " | 240c.c. | 31·4 | +2·5 | 0·9 | 0·257 | 0·37 |
| 47 | " | 66 | 350c.c. | " | 200c.c. | 42·8 | +0·3 | 4·5 | 1·28 | 2·2 |
| 48 | " | 103 | 350c.c. | " | 154c.c. | 56·0 | +0·5 | 3·0 | 0·857 | 1·9 |
| 49 | H. 37 | 44 | 250c.c. | " | 165c.c. | 34·0 | +1·0 | 1·0 | 0·4 | 0·6 |
| 50 | " | 66 | 250c.c. | " | 155c.c. | 38·0 | +1·0 | 1·0 | 0·4 | 0·64 |
| 51 | " | 103 | 250c.c. | " | 135c.c. | 46·0 | +0·75 | 2·0 | 0·8 | 1·4 |

As regards the recovered weights of the bacillary growths. The strain B.C.G. was the only one to show a progressive increase of weight with age, but the older growths of the other strains were generally heavier than the younger ones. An exception to this was found in case of growth from which tuberculin No. 47 was prepared. No adequate explanation for the irregularity of growth weights can be suggested.

## Summary

1. Synthetic media of the chosen formula supported the growth of four mammalian strains of tubercle bacilli, as represented by one virulent human strain, H.37; one virulent bovine strain, Vallée; one modified bovine strain, B.C.G. and one bovine strain of low virulence, No. 110.

2. The acidity of all culture filtrates was less than the original medium, and apparently the age and amount of bacillary growth were responsible for this decrease.

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3. Precipitates were formed in all culture filtrates but one, as a result of concentration. The exception was probably due to the low acidity of the culture filtrate in question.

4. As a general rule the weight of bacillary growth was governed by age, but there were exceptions, which cannot be explained adequately.

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## II. THE ACTIVITY OF TUBERCULIN PRODUCED IN SYNTHETIC MEDIA AS INDICATED BY *IN VIVO* AND *IN VITRO* TEST METHODS

For the determination of potency eleven of the tuberculins prepared and described in Study I were employed and studied by complement fixation, intracutaneous and lethal dose methods. It was considered especially desirable to compare the *in vitro* method with the *in vivo* methods, as Calmette and De Potter (1) found that ordinary glycerinated peptone bouillon culture medium after concentration deviated more units of complement than two tuberculins prepared on Sauton's synthetic medium, according to the complement fixation method employed by them, when combined with an anti-serum obtained from a horse inoculated with B.C.G. Coming from such an authoritative source, this finding has undoubtedly left the impression than the reactions obtained by Watson & Heath (2) and on which their conclusions were based, were non-specific and due to the bouillon content rather than to any specific tuberculin properties of the samples examined.

### Technique

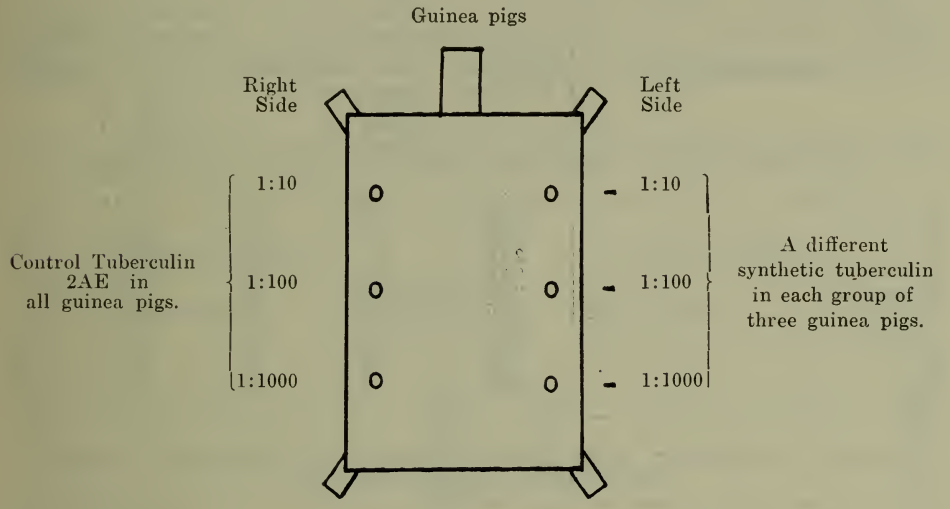
In each of the three methods referred to and employed in this study a tuberculin (K.O.T.), identification 2AE, prepared from cultures of bovine tubercle bacilli on the regular glycerinated peptone beef broth, was included and used as a control for comparison in determinations made with synthetic tuberculins.

#### *Complement Fixation Method*

The method of Watson and Heath (2) was used and was identical with the original, except, of course, that a different anti-serum was employed. The anti-serum was obtained from a mule that had been first inoculated intravenously with two mgs. and five mgs. of a bovine strain of tubercle bacilli of moderate virulence, as judged by guinea pig inoculation, and identified as No. 100, following which an anti-serum rich in antibodies, suitable for the determination of tuberculin potency was produced ten days after the second infection. Subsequently the animal received in the course of the preparation of more antiserum, three intravenous inoculations of 5 mgs. each of the original infective strain, and one of 20 mgs. of another bovine strain, No. 112, of greater virulence, during a period of two years. After a period of rest for two years and five months, 30 mgs. of the original strain No. 100 were again inoculated intravenously and produced a suitable anti-serum identified as H21/Z5H, eleven days later. The anti-serum obtained at this time was ampouled under sterile conditions and kept in the refrigerator and when employed for the determination of tuberculin potency, to be reported in this study, was eight months old.

*Intracutaneous Method*

Thirty-three guinea pigs inoculated subcutaneously, twenty-six days previously with 1 mg. of strain No. 100, were employed. These were divided into eleven groups of three pigs each and a different tuberculin inoculated into the pigs of each group in 0·05 c.c. dose with dilutions of 1:10, 1:100 and 1:1000 of the concentrated tuberculin to be tested, at three different sites of the skin of the left sides of the shaved abdominal and thoracic walls. The control tuberculin 2AE was inoculated intracutaneously at the same time in corresponding doses and dilutions at similar sites on the right side in all pigs of every group for comparison and as indicated below:—




The reactions were recorded at the forty-eighth and seventy-second hours after inoculation, but as these were stronger at the forty-eighth hour with the higher dilutions, the records at this hour were chosen for comparison. In the low dilutions (1:10) reactions were still well marked six days after inoculation. In computing the values of the reactions the following procedure was adopted. The positive reactions were recorded from one plus (+) to four plus (++++); weak positives, as a plus and a minus (+−); and a questionable as a minus and a plus (−+) and a frank negative as a minus (−). The number of plus signs (counting a weak positive as one half plus and a questionable one as one quarter plus) recorded in all dilutions for any single tuberculin in a group of three guinea pigs were added together and compared with the corresponding plus signs recorded for all dilutions of the control tuberculin 2AE in the same group of animals. The deviation from the control was used to determine the potency. This method of computation is admittedly crude, but not more so than recording the length, breadth and thickness of a given reaction, which gives an implied scientific accuracy, which is lacking and often misleading.

*The Lethal Dose Method*

This was essentially the method devised by Schroeder and Brett (3). Nineteen days after the intracutaneous tests the same guinea pigs were employed to test out the potency by the lethal method of ten samples of synthetic tuberculin and of the control 2AE. The various groups of guinea pigs were maintained as in the intracutaneous test and each group of three pigs received intra-abdominally the same synthetic tuberculin as received in the

intracutaneous tests, except the group inoculated with tuberculin 2AE. This group had received in the intracutaneous tests along with 2AE, a synthetic tuberculin of approximately the same strength. For inoculation each synthetic tuberculin employed and the control 2AE were diluted 1:10 from the concentrated product and 2·5 c.c. inoculated for every 500 grammes live weight.

In table 1 is given the results of the intracutaneous method and in table 2, a composite of all three methods, arranged in order of potency by each method.

TABLE 1.—REACTIONS OF INTRACUTANEOUS TESTS

| Group of guinea pigs | Control Tuberculin | Reaction of control tuberculin: Total plus signs at 48th hour | Synthetic tuberculins | Reactions of synthetic tuberculins: Total plus signs at 48th hour | Deviation from standard |
|---|---|---|---|---|---|
| 1 | 2AE | 12¼ | 41 | 13 | + ¾ |
| 2 | 2AE | 12¼ | 42 | 8½ | − 3¾ |
| 3 | 2AE | 17 | 43 | 9½ | − 7½ |
| 4 | 2AE | 16 | 44 | 11¼ | − 4¾ |
| 5 | 2AE | 17½ | 45 | 16½ | − 1 |
| 6 | 2AE | 19½ | 46 | 3½ | −16 |
| 7 | 2AE | 9 | 47 | 15 | + 6 |
| 8 | 2AE | 8½ | 48 | 18¼ | + 9¾ |
| 9 | 2AE | 15¼ | 49 | 8 | − 7¼ |
| 10 | 2AE | 12¾ | 50 | 15¼ | + 2½ |
| 11 | 2AE | 16½ | 51 | 19½ | + 3 |

TABLE 2.—COMPOSITE COMPARISON BY THREE METHODS GIVING ORDER OF POTENCY BY EACH METHOD

| Complement Fixation Method | | Intracutaneous Method | | Lethal Dose Method | | | | |
|---|---|---|---|---|---|---|---|---|
| Tuberculins in order of potency | Unit value per c.c. of dilution | Tuberculins in order of potency | Deviation from standard | Tuberculins in order of potency | Reactions at 24th hour: Dead | Strong | Weak | None |
| No. 51 | 2250 | 48 | + 9¾ | 48 | 3 | – | – | – |
| 48 | 1000 | 47 | + 6 | 51 | 3 | – | – | – |
| 50 | 1000 | 51 | + 3 | 47 | 2 | 1 | – | – |
| 47 | 900 | 50 | + 2½ | 50 | 1 | 2 | – | – |
| 41 | 450 | 41 | + ¾ | 41 | – | – | 3 | – |
| 45 | 300 | 45 | − 1 | 49 | – | 1 | – | 1 |
| 49 | 300 | 2AE | Control | 43 | – | 1 | – | 2 |
| 2AE | 250 | 42 | − 3¾ | 2AE | – | – | 1 | 2 |
| 42 | 200 | 44 | − 4¾ | 42 | – | – | 1 | 2 |
| 44 | 200 | 49 | − 7¼ | 44 | – | – | 1 | 2 |
| 43 | 80 | 43 | − 7½ | 46 | – | – | – | 3 |
| 46 | 50 | 46 | −16 | – | – | – | – | – |
| Concentrated synthetic Media. | ° | – | – | – | – | – | – | – |

## Comment

Consideration of the intracutaneous reactions (table 1) in the various groups of guinea pigs receiving similar doses of the control tuberculin 2AE, will indicate how unevenly the individual groups were sensitized, as the reacting evaluations ranged from a low of eight and one half to nineteen and one half pluses. A further consideration of the values obtained from the synthetic

tuberculins will indicate how easily grave errors might creep into any estimation of potency by the intracutaneous test where adequate control inoculations are lacking.

With the exception of the guinea pigs comprising group 5 (see table 1) all groups received the same synthetic tuberculin in both the intracutaneous and lethal dose methods, as previously stated, yet a review of table 2 will show that almost as much discrepancy exists between the two *in vivo* methods, as between these methods and the complement fixation method. If, however, the tuberculins prepared from the human strain H37, viz. Nos. 49, 50 and 51 are not included in the comparison, it will be found that with the other eight tuberculins, all of which were prepared from bovine strains, an almost perfect agreement between the intracutaneous and complement fixation methods can be established and somewhat less so by the lethal dose method. In fact, there is a closer correlation between the intracutaneous and complement fixation methods for the bovine tuberculins than between the intracutaneous and the lethal dose methods, as a reference to table 3 will indicate.

TABLE 3.—ORDER OF POTENCY OF BOVINE SYNTHETIC TUBERCULINS BY *IN VIVO* METHODS COMPARED WITH THE COMPLEMENT FIXATION METHOD

| Intracutaneous method | | Lethal dose method | |
|---|---|---|---|
| Order of potency | Complement fixation values | Order of potency | Complement fixation values |
| No. 48 | 1000 | No. 48 | 1000 |
| 47 | 900 | 47 | 900 |
| 41 | 450 | 41 | 450 |
| 45 | 300 | 43 | 80 |
| 42 | 200 | 42 | 200 |
| 44 | 200 | 44 | 200 |
| 43 | 80 | | – |
| 46 | 50 | 46 | 50 |

The higher antigenic values given tuberculins Nos. 49, 50, and 51 by the complement fixation method in ratio to their biological activity as compared with tuberculins obtained from strains of bovine origin, were contrary to what might be expected since the anti-serium employed in the titrations was obtained as a result of inoculating tubercule bacilli of bovine origin. On the face of the results obtained it would therefore appear that tuberculins prepared, under the conditions of this study, from the human strain H37, cannot be standardized accurately by the complement fixation method, in comparison with tuberculins prepared from bovine strains.

In comparing the potency of the synthetic tuberculins prepared from all four strains, with the virulence of the strains producing them, or with the period of cultivation, or even with the weights of recovered bacillary growths (table 1, study I) the information obtained did not lend itself to the formulation of any general principles owing to the somewhat conflicting results revealed. However, it was evident that the degree of virulence of a strain of tubercle bacilli was no criterion for the potency of tuberculin it might produce: neither were the weights of bacillary growths in relation to the original volume of media or to the volume of recovered culture filtrates (see study I, table 1) any accurate indication of potency even with the same strain, and the only relative information derived from the evidence given in table 1, study I, was that weight for weight of bacillary growth some strains produce more potent tuberculin than others. There appeared, however to be a limited relation between the age and potency of tuberculins produced from the culture filtrates of virulent strains but not with B.C.G.

Before the publication of the dialysis experiments with tuberculin by Dorset, Henley & Moskey (4), it was generally assumed that the lethal potency of tuberculin was indicative of intracutaneous activity. The results of their experiments, however, have indicated that such an assumption might not be necessarily true, and the question arises, in relation to potency determination of tuberculin by complement fixation, as to which active principle, the intracutaneous or the lethal, is this method referable. Attempts to answer this question will be recorded in studies Nos. 3 and 4.


### Summary

1. The potency of tuberculins prepared from culture filtrates of mammalian strains of tubercle bacilli, including one of human origin and three of bovine origin, cultivated on synthetic media, were determined by the intracutaneous, lethal dose and complement fixation methods
2. Approximately the same amount of variation was found between the intracutaneous and lethal dose methods as between these methods and the complement fixation method for all samples of synthetic tuberculins studied.
3. Titration by complement fixation of synthetic tuberculins prepared from strains of bovine origin only was in full agreement as an indicator of potency with the guinea pig intracutaneous test and with one exception, to the lethal dose method, and with the same exception, the intracutaneous and lethal test results were in accord.
4. Tuberculins prepared from the one strain of human origin revealed higher complement fixation values in ratio to biological activity than did tuberculins prepared from three strains of bovine origin.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3. Schroder, E. C. and Brett, G. W. IBID 1919-54-357.
4. Dorset, M., Henley, R. R., and Moskey, H. E. IBID 1927-71-487.


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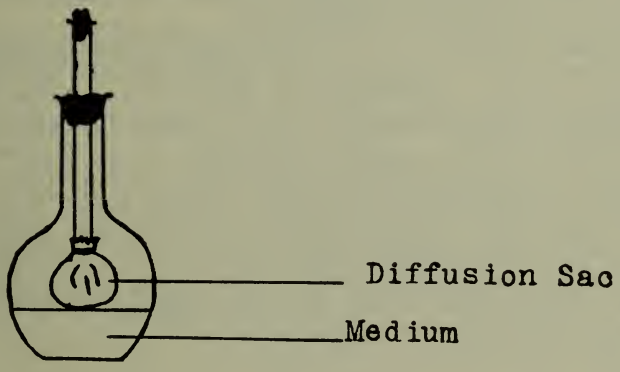
## III. PRELIMINARY DIFFUSION AND DIALYSING METHODS EMPLOYED TO SEPARATE THE ALLEGED INTRACUTANEOUS AND LETHAL PRINCIPLES OF TUBERCULIN

The conflicting reports found in the literature (1) regarding the dialysability of tuberculin may be in part explained by the contribution of Dorset, Henley and Moskey (2) as these authors found, under the conditions of their experiments, that when tuberculin was subjected to dialysis, the dialysate contained mainly the lethal principle while the residue contained the skin reacting principle as well as the lethal principle. Hou (3) on the other hand found that by growing tubercle bacilli in semi-permeable membranes in contact with synthetic medium, the diffusate which became incorporated in the sustaining medium contained tuberculin properties equal in potency to that prepared from surface growths. The methods he employed to determine potency were not stated however.

The work in this study was planned and executed with the view of separating the two alleged active principles of tuberculin by dialysis and to determine which of them might be responsible for the reactions obtained by complement fixation methods. It was first considered desirable, however, to test the accuracy of Hou's (3) findings and to this end the following apparatus was prepared.

A diffusion sac was prepared by cutting a circular piece of cellophane about eight inches in diameter, ballooning it, and then securely attaching the free borders in pleated form to the flared end of a $\frac{5}{8}$-inch bore glass tube about six inches in length. To ensure that the sac was free from leaks it was submerged in water and inflated under suitable pressure. A sac revealing defects was always rejected. A sac having successfully passed this test was freed from excess water and introduced into a 500 c.c. pyrex florence flask containing a predetermined amount of synthetic medium, of the formula as used in study I. The free end of the glass tube forming the orifice of the sac was then plugged with cotton and the tube itself supported in the neck of the flask with another snug cotton plug which was sufficiently loose to permit movement of it up or down but firm enough to hold it at any height to insure that the attached sac might be maintained at any desired position in relation to the surface of the medium.

To prevent contact between the medium and the diffusion sac during sterilization the sac was raised some distance above the surface of the medium by means of its glass connection. Another flask containing a quantity of similar medium was prepared at the same time and both flasks sterilized. In figure 1 is given a diagram of a flask and diffusion membrane as described.




After sterilization and cooling at room temperature a culture from tubercle bacilli strain Vallée was planted in the cellophane sac and the sac pushed in contact with the medium. Another culture from the same strain was floated on the surface of the medium in the other flask. Both flasks were then incubated for one hundred and forty-four days.

At this time both flasks were removed from the incubator. The flask with the surface growth was heated in the autoclave for one hour at 90°C., after which the fluid content was passed through paper and an N. Berkefeld filter, concentrated to one-tenth of the original volume, 0·5 per cent phenol added and identified as No. 58X. In the case of the flask with the cellophane sac, the fluid portion of its contents containing the diffusion products of bacterial growth was removed before the application of heat, brought to the original volume of medium employed with distilled water, passed through an N Berkefeld filter and identified No. 67. It was then ampouled under sterile conditions without a preservative.

The ampouled diffusate was divided into equal portions, one half was left without further treatment and identified as 67A, while the other half was heated in the autoclave for half an hour at 110°C. and fifteen pounds pressure and identified as 67B. This treatment caused 67B to become darker in colour than the unheated portion of the diffusate, 67A. The bacillary growths from both the surface and the diffusion sac were quite good, but slightly more by weight was recovered from the growth in the cellophane, particulars of which will be found in table 1.

TABLE I.

| Identity of tuberculin | Strain | Age of growth | Original volume synthetic medium | Filtrate or diffusate recovered | Dry weight of bacillary growth |
|---|---|---|---|---|---|
| 58X..... | Vallée..... | 144 days. | 250 c.c. | 120 c.c. | 2·5 gms |
| 67..... | " ..... | 144 days. | 250 c.c. | 145 c.c. | 2·75 gms |

There was no occular evidence that any bacilli passed through the diffusion sac, which was corroborated by inoculation of two guinea pigs with 5 c.c. each of the diffusate before filtration.

The potencies of the three samples, viz., 58X, 67A and 67B, were determined by the lethal, intracutaneous and complement fixation methods, as in study II, but with the following modifications.

The guinea pigs employed for the *in vivo* tests were infected with the same strain that produced the tuberculins to be tested, and for the intracutaneous method, only one dilution equivalent to 1:10 of the concentrated tuberculin 58X, was inoculated from each sample. For the lethal dose method the dosage was the same as in study II. In the complement fixation method the pseudo-globulin fraction of serum H21/Z5H was employed instead of the whole serum as was the case in determining the potency of tuberculins 41-51 (study II). This fraction of anti-serum although capable of determining potency of tuberculins gave values of about one half that obtained with the whole serum as the following table 2 will indicate, so that in any comparison of the complement fixation values of tuberculins 41-51, with the tuberculins in this study, due recognition should be given to this variation.

TABLE 2.

| Crude tuberculin | Unit value with | |
|---|---|---|
| Identity | Whole anti-serum H21/Z5H | Pseudo-globulin fraction H21/Z5H |
| No. 48..... | 10,000 | 5,000 |
| 47..... | 9,000 | 4,000 |
| 43..... | 80 | 30 |
| 42..... | 200 | 100 |

In table 3 will be found the results of comparative tests for potency by the three methods of the tuberculin samples 58X, 67A and 67B.

TABLE 3.

| Identity of tuberculin | Complement Fixation Method | Lethal Dose Method | | | | | Intra-cutaneous Method |
|---|---|---|---|---|---|---|---|
| | Values per c.c. of inocula | Guinea pigs tested | Reactions at 24th hour | | | | Average reactions in three guinea pigs |
| | | | Died | Strong | Weak | None | |
| 58X | 200 | 3 | 2 | – | 1 | – | +++ |
| 67A | 175 | 3 | 2 | – | 1 | – | +++ |
| 67B | 175 | 3 | 3 | – | – | – | +++ |

The results obtained and as given in table 3 indicated that under the conditions of the experiment the diffusate, unheated or heated, as represented by tuberculins 67A and 67B of tubercle bacilli, strain Vallée, during growth, contained both the skin reacting and lethal principles, *in vivo* and complement fixing bodies *in vitro*, and in comparative potency tests by these test methods were practically identical to a corresponding tuberculin, No. 58X, prepared in the conventional manner from the same strain and confirmed Hou's conclusions (3).

It is quite obvious, however, that this diffusion method did not meet the requirements for separating the two alleged active principles, therefore dialysis under other conditions were attempted with:—

(*a*) Tuberculins prepared under different conditions as represented by tuberculins 57X, 67A and 67B.

(*b*) Tuberculins in the course of production.

(*a*) *Dialysis of tuberculins prepared under different conditions*

Three dialysing sacs of cellophane prepared similar to the one previously described, but somewhat smaller, were placed individually in three one-litre Erlenmeyer flasks containing 750 c.c. of distilled water each and sterilized. The usual precaution was taken to prevent contact between the dialysing sacs and the distilled water in the flasks during sterilization. After cooling, in one sac was placed 5 c.c. of concentrated tuberculin 58X, representing a tuberculin prepared from a culture filtrate in the conventional manner; in another 25 c.c. of tuberculin 67A and in the third, 25 c.c. of tuberculin 67B, representing unheated and heated tuberculin samples obtained from the diffusion products of tubercle bacilli during growth. The dialysing sacs were then pushed in contact with the distilled water and dialysis allowed to continue at room temperature for three weeks, after which all three sacs were removed. The following observations, as given in table 4, were recorded in connection with the residual fluids in the three sacs.

TABLE 4.

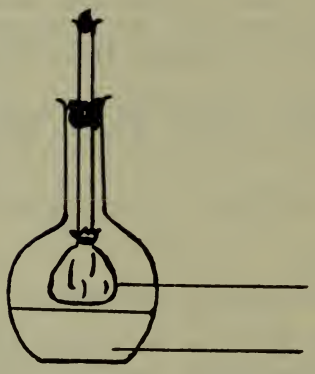
| Original contents of dialysing sacs. | Volume recovered | Remarks |
|---|---|---|
| 5 c.c. Tuberculin 58X | 15 c.c. | Fluid turbid with trace of original colour. |
| 25 c.c. diffusate 67A | 37 c.c. | Fluid clear with trace of original colour. |
| 25 c.c. diffusate 67B | 32 c.c. | Fluid opalescent with trace of original colour. |

The residual fluids in the dialysing sacs from tuberculins 58X and 67B respectively were made to 37 c.c. each with distilled water to correspond in volume to the residual fluid of tuberculin sample 67A, and the dialysates in the three flasks were concentrated separately to 37 c.c. each. Practically all the original colouring matter of the samples was contained in the dialysates and during concentration they gave off the characteristic odour of tuberculin.

(*b*) *Dialysis of tuberculin in the course of production*

For this method two florence flasks were prepared in the same manner as the Erlenmeyer flasks for use at room temperature, but with the following modifications. In place of 750 c.c. of distilled water only 200 c.c. were used in each of these (see figure 2) and each cellophane sac received 150 c.c. of synthetic media of the formula previously used. Both flasks were then sterilized, taking the precaution not to allow contact between the cellophane sacs and the distilled water. There was no evidence of the escape of any of the media from the sacs.

Figure 2




The same strain of tubercle bacilli employed to produce tuberculins 58X and 67 (A and B), Vallée, was planted on the surface of the media in both dialysing sacs after sterilization and cooling. One of the sacs was at once pushed in contact with the distilled water and both flasks placed in the incubator for seventy-one days. At the fiftieth day of incubation, contact between the dialysing sac and distilled water was made in the second flask. In other words, in one flask dialysis was continuous during the entire period of growth while in the other only during the last three weeks of growth.

On the seventy-first day both flasks were removed from the incubator, contact between the sacs and surrounding fluids broken, and heated in the autoclave for one hour at 90° C. The contents of both sacs were passed through paper separately and the volume of each recorded. The insides of the sacs were then washed out with distilled water and these washings passed through paper and added to their corresponding culture filtrates. The fluids, made up of these combined paper filtrates—culture filtrates and washings, were then passed separately through N Berkefeld filters and each concentrated over a water bath to one-tenth of the original volume of medium. Phenol was added to each as a preservative in 0·5 per cent amounts. The corresponding dialysates without filtration were concentrated to 15 c.c. and 0·5 per cent phenol added.

The culture filtrate from the cellophane sac which had dialysed over the entire period of growth was identified as 63 after concentration and its correspond-

ing concentrated dialysate as 63A, while the other culture filtrate which had dialysed only during the last three weeks of growth was identified as 64 after concentration, and its corresponding concentrated dialysate as 64A. The dialysates from both flasks after concentration were much darker in colour than their corresponding residues and the characteristic odour of tuberculin was pronounced in them. Flocculent precipitates were present in 63 and 63A after concentration, while in case of the other samples, 64 and 64A. a precipitate was present only in the former. In table 5 will be found particulars regarding the four preparations.

TABLE 5.

| Original fluid in | | Fluid recovered from | | Period of Dialysis | Dry weight of growth recovered | Identity of concentrated | |
|---|---|---|---|---|---|---|---|
| Dialysing sac | Flask proper | Dialysing sac | Flask | | | | |
| Media | Distilled water | | | | | Filtrate | Diffusate |
| 150 c.c..... | 200 c.c. | 50 c.c. | 190 c.c. | Whole period of growth—71 days. | 1·65 | 63 | 63A |
| 150 c.c..... | 200 c.c. | 50 c.c. | 190 c.c. | Last 21 days of growth. | 1·45 | 64 | 64A |

The residues and dialysates obtained as described and the corresponding undialysed tuberculins were subjected to potency tests by the complement fixation, lethal dose and intracutaneous methods, which were identical to those previously described in this study (see table 3). In table 6 will be found the results.

## Comment

A review of table 6 will reveal that the dialysable fraction of No. 58X, a tuberculin prepared in the conventional manner from a culture filtrate of a surface growth, contained the same active principles, although in a lower degree, as its residue as indicated by intracutaneous lethal and complement fixation test methods. On the other hand, the dialysable fractions of Nos. 67A and 67B, unheated and heated samples of a tuberculin produced by diffusion of metabolic product of bacterial growth through a cellophane membrane, differed quite markedly, both in kind and degree as regards potency from their non-dialysable fractions by intracutaneous and lethal dose test methods. Likewise, the complement fixation activities of the former were extremely low in comparison with those of the latter. Moreover, the dialysates of these two samples differed one from another in that the dialysable fraction from the heated sample, 67B, contained more lethal activity and higher complement fixation value than the unheated sample, 67A. To further complicate matters, the dialysates, 63A and 64A of the two tuberculins which were dialysed in the course of production were without any trace of activity by *in vivo* test methods, while indicating a small degree of complement fixation value.

TABLE 6.

| Identity of inocula employed | Complement Fixation Method | Lethal Dose Method | | | | | Intracutaneous Method |
|---|---|---|---|---|---|---|---|
| | | | Reactions at 24th hour | | | | |
| | Values per c.c. of inocula | No. guinea pigs tested | Died | Strong | Weak | None | Average reactions in three guinea pigs |
| Tuberculin 58X | 200 | 3 | 2 | – | 1 | – | +++ |
| Residue after 3 weeks dialysis at room temperature | 125 | 3 | – | 2 | 1 | – | +++ |
| Dialysate after 3 weeks at room temperature | 50 | 3 | 1 | 1 | 1 | – | ++ |
| Diffusate 67A | 175 | 3 | 2 | – | – | 1 | +++ |
| Residue after 3 weeks dialysis at room temperature | 175 | 3 | 3 | – | – | – | +++ |
| Dialysate after 3 weeks at room temperature | 5 | 3 | – | – | 1 | 2 | – |
| Diffusate 67B | 175 | 3 | 3 | – | – | – | +++ |
| Residue after 3 weeks dialysis at room temperature | 100 | 3 | 3 | – | – | – | +++ |
| Dialysate after 3 weeks at room temperature | 25 | 3 | – | 1 | 1 | 1 | – |
| Tuberculin 63 | 100 | 3 | 3 | – | – | – | +++ |
| Dialysate during 71 days growth, 63A | 10 | 3 | – | – | – | 3 | – |
| Tuberculin 64 | 100 | 2 | 2 | – | – | – | +++ |
| Dialysate during last 3 weeks of growth, 64A | 7½ | 2 | – | – | – | 2 | – |

These contradictory results obtained as a result of potency determinations by intracutaneous, lethal and complement fixation test methods of the dialysable fractions of the various tuberculin samples studied, precluded the formulation of definite conclusions concerning the objects of the experiment; although it is quite obvious that the major portion of the principle responsible for complement fixation reactions is contained in the residues.

## Summary

1. The products of tubercle bacilli during growth which diffuse through a semi-permeable membrane in contact with suitable synthetic media are incorporated in the sustaining medium forming a product indistinguishable from a concentrated tuberculin prepared in the conventional manner from the same strain, as determined by intracutaneous and lethal methods *in vivo* and complement fixation *in vitro*.

2. Concentrated tuberculin prepared in the usual manner from a culture filtrate of a surface growth of tubercle bacilli on synthetic medium permitted a percentage of both the skin reacting and lethal principles and one quarter of the original complement fixing substances to pass through a semi-permeable membrane when dialysed.

3. A tuberculin prepared from the diffusion products of tubercle bacilli, unaltered by either heat or chemicals, permitted a fraction indicating lethal activity and insignificant amounts of complement fixing properties to pass through a dialysing membrane. The application of heat under pressure appeared to enhance the passage of a greater percentage of the fraction with lethal activity, as well as more complement fixing properties.

4. Concentrated tuberculins prepared from culture filtrates which had dialysed during the entire period of growth of bacilli or during the last three weeks only as described, revealed neither intracutaneous or lethal activity in their dialysates, as indicated by *in vivo* tests, although revealing low complement fixing properties.

5. The volatile elements of tuberculin dialysates, with one exception, as represented by the characteristic odour and associated with the colouring matter, appear to be practically devoid of the skin reacting properties, although containing a small percentage of complement fixing bodies, and also indicating lethal activity.

6. The methods of diffusion or dialysis employed did not offer a sharp separation of the alleged skin reacting and lethal principles, and in fact, the former permitted the diffusion of both intracutaneous and lethal principles; but there is some evidence to show that the fraction which is dialysable as against that which is diffusible contains proportionately more of the lethal principle and less of the complement binding bodies, and that the non-dialysable fraction contains proportionately more of the skin reacting principles and correspondingly more complement fixing bodies.

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## IV. THE ACTIVITY OF RESIDUES AND DIALYSATES OF TUBERCULIN WITH ESPECIAL REFERENCE TO INTRACUTANEOUS, LETHAL AND COMPLEMENT FIXATION REACTIONS

In the course of determining potency of tuberculins prepared from culture filtrates of tubercle bacilli grown on synthetic media, one sample, identified as No. 80, was encountered, which was very potent by the lethal dose method but only gave very low values in comparison by complement fixation titration. Intracutaneous tests, however, with this tuberclin in tuberculous guinea pigs at periods of from thirty-one to ninety-nine days after infection varied considerably, with results ranging from negative to three plus reactions; and the positive reactions appeared to correspond roughly to the length of time the animals were infected. In contrast, control inoculations in the same animals with another tuberculin, No. 86, always gave frank positive reactions by the intracutaneous test. This tuberculin, like No. 80, was also very potent by lethal dose methods, but, unlike it, had a high complement fixation value. In the following table 1 will be found the results of intracutaneous inoculations of both these tuberculins made in tuberculous guinea pigs after different periods of infection. The tests were made over a period of eighteen months in five groups of test animals inoculated with the same infective strain.

Tuberculin No. 80 was prepared from the culture filtrate of a surface growth of strain Vallée grown on synthetic media. The method of preparation differed from the usual procedure in that the flask containing the growth had been overlooked and had remained in the incubator for over eight months, after which it was sterilized in the autoclave under pressure and then kept at room temperature for another two months before it was finally filtered, concentrated and preserved with 0·5 per cent phenol. Soon after concentration the antigenic value as determined by complement fixation titration was 2,000 units per c.c. of crude but there was a progressive drop in the value with age and about two years after concentration the antigenic value had practically vanished.

TABLE 1

| Period of infection before test | No. of guinea pigs tested | Intracutaneous reactions with | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Tuberculin No. 80 guinea pigs tested | | | | Tuberculin No. 86 guinea pigs tested | | | |
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 36 days..... | 2 | + | − | not tested | | ++++CN* | +++ CN | not tested | |
| 39 days..... | 4 | − | −B† | − | − | ++++CN | ++++ CN | +++ CN | +++ CN |
| 51 days..... | 2 | − | + | not tested | | ++++CN | ++++ | not tested | |
| 61 days..... | 3 | +++ | + | − | not tested | ++++ | ++++ | ++++ | not tested |
| 99 days..... | 3 | +++ | ++ | − | " | ++++ | ++++ | ++++ | " |

CN* = Central Necrosis. + = Positive.
B† = Blanching. − = Negative.

This tuberculin No. 80 in conjunction with two others, Nos. 86 and 89, prepared from culture filtrates of surface growths of strain No. 110 in the usual manner, were selected for dialysis. No. 86 had been grown for one hundred and fifty days and No. 89 for two hundred and six days on the same kind of synthetic medium. They were only subjected to a temperature of 90° C. for one hour before concentration. The dry weight of the bacillary growth recovered from each was 1·5 grammes and the complement fixation values were 20,000 and 15,000 respectively when originally determined approximately two months in the case of No. 86 and two weeks in the case of No. 89, after concentration, but at a later period, approximately eighteen months after the first tests, values of 20,000 and 8,000 were obtained for Nos. 86 and 89 respectively. The determination in both instances were made with the same antiserum, H83/Z 1A, obtained from a horse on the thirteenth day following a re-infection of a bovine strain of tubercle bacilli, No. 100. In Table 2 is given the results of the first series of tests made at the same time with tuberculins Nos. 80, 86 and 89 by complement fixation and lethal dose methods and also the results of complement fixation, lethal dose and intracutaneous methods of a second series of tests at the time they were selected for dialysis. At this time there was a further drop as compared with the first tests in the antigenic values of tuberculins Nos. 80 and 89.

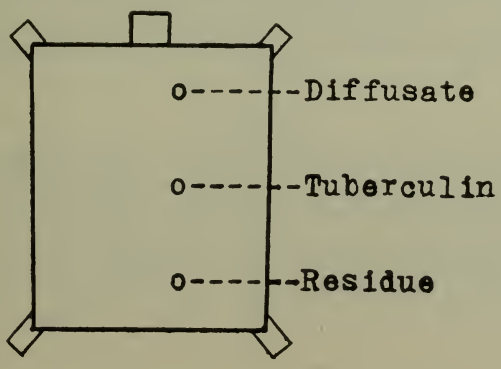
TABLE 2.

| Test | Identity of tuberculin | Complement fixation method | Lethal Dose Method | | | | | Intracutaneous method |
|---|---|---|---|---|---|---|---|---|
| | | Value of crude | No. of guinea pigs tested | Reactions at 24th hour | | | | Average plus signs in three guinea pigs |
| | | | | Died | Strong | Weak | None | |
| First series..... | 80 | 50 | 5 | 4 | 1 | – | – | Not tested |
| | 86 | 20,000 | 5 | 4 | 1 | – | – | " |
| | 89 | 15,000 | 5 | 3 | – | – | 2 | " |
| Second series at time selected for dialysis. | 80 | 25 | 5 | 4 | – | 1 | – | – |
| | 86 | 20,000 | 5 | 4 | – | – | 1 | ++++ |
| | 89 | 6,000 | 5 | 5 | – | – | – | ++ |

For dialysis three flasks were prepared as for the tuberculin samples 58X 67A and 67B in study III and in each dialysing sac was placed 3 c.c. of a different samples of concentrated tuberculin of the three selected and contact made between the water and the dialysing sac. After six days dialysis the individual dialysates were removed and replaced by 750 c.c. of sterile distilled water, and dialysis was allowed to continue for twelve more days. The first dialysates were separately concentrated immediately after removal to 15 c.c. and phenol added as a preservative. The second lot were also concentrated on removal to about the same volume (15 c.c.) and the dialysates obtained from each tuberculin mixed together and finally concentrated to 25 c.c. All of the dialysates contained the major portion of the colouring matter and when concentrated gave off the characteristic odour associated with tuberculin. The residual fluids in the three sacs, measured 19 c.c. 25 c.c. and 13 c.c. from tuberculins Nos. 80, 86 and 89 respectively. All of them were made to 25 c.c. and were almost colourless.

*In Vivo Potency Tests*

The residues, the dialysates and equal parts of residues and diffusates of each tuberculin without further dilution were inoculated intra-abdominally into infected guinea pigs in a dose of 2·5 c.c. per 500 gms. live weight for the lethal dose method. For the intracutaneous method a group of three sensitized guinea pigs was employed for each residue, dialysate and their corresponding undialysed tuberculin—this latter in the dilution 3:25 to approximate the dilution of the residue and dialysate—served as control and each animal of a group received intracutaneously without further dilution a dose of 0·05 c.c. of each product at a different site in the skin on the thoracic and abdominal walls after removal of the hair by shaving, as indicated in the following diagram:—



*In Vitro Potency Test*

For the complement fixation, the usual technique of Watson & Heath (3) was employed for titrating the residues, dialysates and the undialysed concentrated tuberculins. These latter, diluted as for the intracutaneous test, 3:25, served as controls. The anti-serum employed was from the same animal that produced it for the two first tests in this study, viz., Horse No. 83, but it was obtained as a result of another intravenous re-infection and was identified H83/Z1I and when employed in this test was ten days old. The re-infection was made intravenously with 15 mgs. of the bovine strain No. 205, suspended in saline and the anti-serum H83/Z1I was collected eleven days after reinfection. In table 3 will be found the results.

Comment

Although table 3 is lacking in some details the results given show that under the method of dialysis employed in this study, the dialysates in every instance contained *only* the lethal principles by *in vivo* test methods and in every case was without value by complement fixation titration. On the other hand, the residues of the two tuberculins with definite skin reacting principles contained both the skin reacting and lethal principles, but the former principle in a diminished amount as compared with the undialysed samples and the latter principle in a diminished amount as compared with the dialysates. The residue also contained complement fixing bodies but were diminished approximately by half as compared to the undialysed samples.

These results support the findings of Dorset, Henley & Moskey (1) and further indicate that the complement fixing property is associated chiefly with the skin reacting principle, although it may also indicate the lethal potency of tuberculin, provided that no great amount of hydrolysis has occurred, for once the two principles are separated they apparently cannot be reunited by simple mixing, and such mixtures either mask or reduce the complement fixing reactions of the residues, as reference to table 3 will indicate, and is quite different from fractions that might be obtained by alcohol fractionation, as will be shown in study VII.

The hypothesis of the above authors (1) that the lethal principle *per se* and as a separate entity, is formed as a result of the hydrolysis of a complex molecule containing both the skin reacting and lethal principle, resulting in tuberculins with various graduations of the dialysable lethal principle, appears to be correct; and it would seem as if the amount of the specific dialysable lethal principle present in any particular sample of tuberculin will be dependent on the degree of hydrolysis it had undergone and its lethal activity will be in inverse ratio to its skin reacting power.

TABLE 3

| Product | Complement fixation method | Lethal Dose Method | | | | | Intracutaneous method |
|---|---|---|---|---|---|---|---|
| | | | Reactions at 24th hour | | | | |
| | Value per c.c. of inocula | Guinea pigs tested | Died | Strong | Weak | None | Average reactions in three guinea pigs |
| Tuberculin No. 80 | 15 | Not tested at this time | | | | | ++ |
| Dialysate of 80 | Negative | 5 | 3 | 1 | 1 | – | – |
| Residue of 80 | 15 | 5 | 1 | 1 | – | 3 | ++ |
| ãã Dialysate and residue | Negative | 5 | 2 | – | 2 | 1 | Not tested |
| Tuberculin No. 86 | 1250 | Not tested at this time | | | | | ++++ |
| Dialysate of 86 | Negative | 5 | 4 | – | 1 | – | – |
| Residue of 886 | 600 | 5 | 3 | – | – | 2 | ++ |
| ãã Dialysate and residue | 150 | 5 | 4 | – | – | 1 | Not tested |
| Tuberculin No. 89 | 500 | Not tested at this time | | | | | +++ |
| Dialysate of 89 | Negative | 5 | 4 | 1 | – | – | – |
| Residue of 89 | 300 | 5 | 3 | – | – | 2 | ++ |
| ãã Dialysate and residue | 50 | 5 | 4 | 1 | – | – | Not tested |

These same authors in another contribution (2) showed that age in reference to the period the bacilli were in contact with the sustaining medium, was at least a contributing factor in determining the amount of the specific and dialysable lethal principle found in a tuberculin. This has also been indicated in this study, for tuberculin No. 80, the oldest of the three tuberculins on the basis stipulated, was almost entirely composed of the lethal principle, while No. 89 the next oldest, contained more of the lethal principle not in combination with the skin reacting principle as indicated by the complement fixation titration than did No. 86 the youngest of the three. It is also quite evident that a tuberculin like No. 80 would be inadequate by the intracutaneous method for diagnostic purposes, particularly in early tuberculosis but would be considered fully potent if standardized by the lethal method only.

It should be noted that the antigenic values given tuberculins Nos. 80, 86 and 89 were diminished roughly by one half at the time inoculated into guinea pigs in conjunction with their respective residues and dialysates (see table 3) as compared with the values obtained approximately one month previously when these tuberculins were selected for dialysis (see table 2). The reduction in values was probably due to the quality of the anti-serum used, H83/Z1I, for six months later it was found to be practically worthless for determining tuberculin potency. Although it was unfortunate that no more of the original anti-serum, H83/Z1A, was available in the final test, the discrepancies in the evaluations given by the two antisera have no direct bearing on the results of this experiment.

## Summary

1. A tuberculin prepared from a culture filtrate of a surface growth of tubercle bacilli grown on synthetic medium for a period of over eight months was found to be almost totally deficient in the skin reacting principle in early tuberculosis of guinea pigs and also with a very low antigenic value by complement fixation titration, but gave weak and erratic skin reactions in guinea pigs with disease of longer standing. It was, however, very potent by the lethal dose method.

91845—4½

2. Under the conditions of dialysis in this experiment the dialysates of the three tuberculins studied contained only the lethal principle and did not reveal antigenic values by complement fixation titration, while the residues of the tuberculins with skin reacting activity contained in addition lethal principles and also complement binding substances.
3. The dialysable lethal principle on the one hand and the non-dialysable skin reacting principle (containing the lethal principle also) on the other, cannot be re-united after separation by simple mixing, and, in fact, such mixing apparently masks or reduces the antigenic properties capable of deviating complement that were originally present in the latter.
4. A tuberculin such as No. 80 would be quite inadequate for use in the diagnosis of early tuberculosis by the intracutaneous method although it would be considered almost superpotent by the lethal dose method of standardization.
5. The antigen responsible for the complement fixation reaction in the residue of tuberculin after dialysis appears to be represented by or identical with the cutaneous-lethal complex and distinct from the lethal principle as a separate entity.

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## V. THE ACTIVITY AS INDICATED BY LETHAL AND COMPLEMENT FIXATION METHODS OF TUBERCULINS PRODUCED IN SYNTHETIC MEDIA CONTAINING (*a*) AMMONIUM SUCCINATE (*b*) ASPARAGIN, AS SOURCES OF NITROGEN

The results of previous studies (1 and 2) revealed that potent tuberculins could be prepared from culture filtrates of tubercle bacilli grown on synthetic medium containing asparagin as the source of nitrogen. Asparagin, however, is relatively expensive and in search for some less expensive form of nitrogen that might be utilized to replace this reagent, ammonium succinate was found in a formula for synthetic medium described by Gessard and Vaudremer (1). Eberson (2) employing this medium found that tubercle bacilli would grow on it without glycerine, but that more luxuriant growths were obtained when 2 per cent glycerine was added; moreover, he was able to prepare potent tuberculin from the culture filtrates.

Preliminary experiments corroborated the findings of Eberson as regards growth, in that tubercle bacilli did propagate on this medium without glycerine but growth was extremely slight and filmy. The addition of 2 per cent glycerine improved the growth and 5 per cent glycerine caused quite a profuse growth, which climbed the sides of the containers in which the organisms were grown, but growth remained rather filmy in structure.

For the study under consideration two batches of media were prepared with the following ingredients:—

| No. 1 | | | No. 2 | | |
|---|---|---|---|---|---|
| Gessard and Vaudremer's (1) | | | Dorset, Henley & Moskey's (3) | | |
| Ammonium succinate | 5·0 | gms. | Asparagin | 5·0 | gms. |
| Dipotassium phosphate | 5·0 | " | Dispotassium phosphate | 1·0 | " |
| Magnesium sulphate | 2·5 | " | Magnesium sulphate | 1·0 | " |
| Calcium chloride | 1·25 | " | Sodium citrate | 0·5 | " |
| Glycerine | 50 | c.c. | Ferric ammonium citrate | 0·075 | " |
| Distilled water to | 1000 | c.c. | Glycerine | 60 | c.c. |
| | | | Distilled water to | 1000 | c.c. |

Each medium was distributed in four flasks in 250 c.c. amounts and without any adjustment, sterilized. After sterilization the same strains of tubercle bacilli, as previously utilized for the production of tuberculins in study I, viz., H37, B.C.G., Vallée and No. 110, were planted in duplicate on each medium, that is, from an original culture on solid synthetic medium of each strain, a sub-culture was made first on one medium and then on the other. The results from study II indicated that stronger tuberculins were more generally produced at the sixty-sixth and one hundred and third days of growth than at the forty-fourth day, so that these cultures were therefore allowed to remain in the incubator for 116 days. At this time, all eight flasks were removed from the incubator, heated in the autoclave for one hour at 90°C. and the growths separated individually by filtration on Whatman No. 2 filter paper. The paper filtrates were finally passed through an N Berkefeld filter, concentrated to one-tenth of the original volume of medium and 0·5 per cent phenol added. The weight of the individual cultures were determined after drying in the incubator for 96 hours.

Sixty-two days after concentration the potency of all eight tuberculins were determined by the complement fixation method of Watson & Heath (4) and seven of them by the lethal method of Schroder and Brett (5). The techniques were as described in study II. In table 1 will be found the results along with pertinent information regarding the eight tuberculins. The results of potency determinations were practically the same by both methods of tests and it is regrettable that the intracutaneous method was not used comparatively also.

To further check the parallelism between the two methods employed, four of the eight tuberculins were selected for further study, viz., Nos. 68, 69, 72 and 74, and were the products of three different strains. Two of these tuberculins, Nos. 68 and 74, prepared from the culture filtrates of the bovine strains, Vallée and No. 110, respectively, on medium No. 2, might be considered super-potent as revealed by the original lethal dose method; one, No. 72, from the culture filtrate of the human strain, H37, on No. 2 medium, as of average potency and the fourth, No. 69, from the culture filtrate of the bovine strain, Vallée, on No. 1 medium, as sub-potent.

These four tuberculins were diluted with distilled water so that each c.c. of the diluted tuberculin contained 1000 units, or, in other words, as follows: No. 68, 1:20; No. 69, $2\frac{1}{2}$:3; No. 72, 1:10 and No. 74, 1:30. Tuberculous guinea pigs infected at the same time as in the original test for potency were inoculated intra-abdominally with 2500 units or 2·5 c.c. of the diluted tuberculins per 500 grammes live weight. The results will be found in table 2.

TABLE 1

| Identity of tuberculin | T.B. strain | Medium | Amount culture filtrate recovered in c.c. | Dry Wt. bacillary growth recovered in grammes | Value by complement fixation method per 1c.c. crude | Lethal method | | | | | Order of potency by | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | No. guinea pigs tested | Reactions at 24th hr. | | | | C.F. method | Lethal method |
| | | | | | | | Died | Strong | Weak | None | | |
| No. 68.... | Vallée.. | No. 2... | 130 | 2·55 | 20,000 | 5 | 4 | 1 | ..... | ..... | 2 | 2 |
| No. 69.... | " .. | No. 1... | 155 | 0·65 | 1,200 | 5 | 1 | ..... | 2 | 2 | 5 | 5 |
| No. 70.... | B.C.G. | No. 2... | 140 | 2·35 | 2,500 | 5 | 2 | 1 | ..... | 2 | 4 | 4 |
| No. 71.... | " .. | No. 1... | 155 | 0·025 | 75 | | not | tested | | | | |
| No. 72.... | H37.... | No. 2... | 140 | 2·00 | 10,000 | 5 | 3 | ..... | 1 | 1 | 3 | 3 |
| No. 73.... | " .. | No. 1... | 165 | 0·15 | 1,000 | 5 | ..... | ..... | 3 | 2 | 6 | 6 |
| No. 74.... | No. 110. | No. 2... | 150 | 2.15 | 30,000 | 5 | 5 | ..... | ..... | ..... | 1 | 1 |
| No. 75.... | " .. | No. 1... | 100 | 0·175 | 750 | 5 | ..... | ..... | 3 | 2 | 7 | 7 |

TABLE 2

| Identity of tuberculin | Dilution | Units per c.c. of dilution | No. guinea pigs tested | Lethal method | | | |
|---|---|---|---|---|---|---|---|
| | | | | Reactions at 24th hr. | | | |
| | | | | Died | Strong | Weak | None |
| No. 68 | 1 : 20 | 1,000 | 5 | 3 | 1 | – | 1 |
| No. 69 | 2½: 3 | 1,000 | 5 | 2 | – | – | 3 |
| No. 72 | 1 : 10 | 1,000 | 5 | 2 | – | 1 | 2 |
| No. 74 | 1 : 30 | 1,000 | 5 | 3 | – | 1 | 1 |

N.B.—Note difference in lethal results with tuberculin No. 72, in Tables 1 and 2, although dosage in each case was the same.

## Comment

The cultures of all four strains grew well on No. 1 medium covering the surface of the medium and even climbing up the sides of the flasks in which they were grown, but remained filmy in form, and as shown by the dry weights of bacillary growths recovered (table 1) were considerably less than the same strains grown on No. 2 medium. Within certain limits, the recorded evidence indicated, as in study II, that the weight of bacillary growth was indicative of potency, and perhaps can be accepted as a general rule, with the reservation that certain strains produce more potent tuberculin than do others.

Theoretically, virulent strains grown for the same period of time on the same medium under the same conditions, should produce more potent tuberculins than avirulent or attenuated strains, and generally the evidence in this study supported this view, as a comparison between tuberculins Nos. 68 and 70, produced respectively by strain Vallée (virulent) and by B.C.G. (attenuated) will indicate. Nevertheless, it would appear that certain strains (e.g. No. 110 of very low virulence), regardless of virulence, produced more potent tuberculin, weight for weight of bacillary growth, than did others.

The antiserum employed to titrate the various tuberculins was not the same as used in study II, although produced by the same animal, Mule No. 21. The antiserum in question, H21/Z5S, was obtained ninety days after the last intravenous inoculation of 30 mgs. of live tubercle bacilli, bovine strain, No. 100, and ten days after the intracutaneous inoculation of eight of the synthetic tuberculins prepared in study I, which resulted in an increase in serum antibodies. When employed for the titration of the tuberculins, Nos. 68-76, it was over two years old.

It will be noted that the complement fixation values of tuberculins, Nos 72 and 73, prepared from strain H37 in this study, conformed to biological potency in relation to the other tuberculins prepared from organisms of bovine origin and differed in this respect from the results obtained with tuberculins 49, 50 and 51 prepared from this same human strain in study II. The cause of the variation with the first three samples, Nos. 49, 50 and 51, prepared from H37 is unknown and no adequate explanation can be offered, although it must be considered that anti-sera used in the two studies were prepared differently and may or may not have some bearing on the matter.

In this study, at least, the complement fixation method compared very favourably with the lethal dose method as regards the order of potency and this agreement was further supported by the results in table 2. In the previous study, No. IV, however, the evidence presented supported the skin reacting principle as the fraction causing the complement fixation reactions. The conflict, however, between the results of this study and study IV, is only apparent because the molecule containing the skin reacting principle is in itself capable of causing the lethal reaction, whereas, that containing the purely lethal principle appears to be incapable of producing the skin reaction in early tuberculosis, at least. The close agreement between the two methods employed in this study was undoubtedly a coincidence perhaps due to the similarity of technique employed in the preparation of all eight tuberculins, or at the time tested, either no hydrolysis had occurred in any of the samples or the same degree of it was present in them all.

Long (6) in criticising the original work of Watson & Heath (4), suggested that the close agreement between the complement fixation and lethal dose method —this latter as conducted by Schroeder—was nothing more than a coincidence, which was nearer the truth than he suspected at that time, for his remark was based on a false premise, viz., Calmette's statement that purified tuberculin will not serve as an antigen, the incorrectness of which has been already indicated in studies III and IV, in both of which the residues of dialysed synthetic tuberculins were shown to have antigenic values by complement fixation tests. Further evidence in rebuttal of this statement will be found in study IX.

## Summary

1. Filtrates of four strains of tubercle bacilli grown on synthetic media containing ammonium succinate as the source of nitrogen did not produce tuberculins comparable in potency to filtrates of the same strains grown on synthetic medium containing asparagin as the source of nitrogen.

2. Close agreement regarding potency was obtained between the complement fixation and lethal dose methods, but this should not invalidate the evidence presented in study IV regarding the principle responsible for the complement fixation reactions, since the agreement in this study was considered a coincidence due to the similarity of the methods employed in the preparation of the tuberculins, resulting in little or no hydrolysis in the individual samples.

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## VI. THE INDICATED ACTIVITY BY *IN VIVO* AND *IN VITRO* TEST METHODS OF TUBERCULINS PRODUCED WITHIN AND DIFFUSING THROUGH A CELLOPHANE SAC IN CONTACT WITH SYNTHETIC MEDIA IN THE COURSE OF CONTINUOUS GROWTH

As shown in study III potent tuberculin could be produced by cultivating tubercle bacilli in a diffusion sac of cellophane in contact with suitable synthetic medium and confirmed the work of Hou (1). The experimental work to be reported in this study was planned with a view to study, by *in vivo* and *in vitro* methods the potency of tuberculins prepared from the diffusates of a single culture over a long period of time. For the requirements of the experiment, some special apparatus was necessary and was prepared in the following manner.

### PREPARATION OF APPARATUS

*Flask "A."*—A round hole was made in a 500 c.c. florence pyrex flask above the 200 c.c. level, with the aid of an ordinary grinding stone and a rat tail file and in it was inserted a stopcock supported by a rubber stopper. The stopcock was further supported by a string attached to the neck of the flask. A circular piece of cellophane eight inches in diameter was tied to the flared end of a piece of glass tubing, six inches long by five-eighths of an inch in diameter, in a manner to form a sac, as previously described in study III. After ascertaining that no air could pass when the sac was submerged in water and inflated by blowing into it, the free end of the glass tube was plugged with cotton and the sac introduced into the prepared florence flask. A further piece of glass tubing, one quarter-inch bore, was bent U-shaped, one arm being short enough to allow of insertion into the flask and the length of the base connecting the arms of the U about one inch longer than the neck of the flask. This was also introduced, short arm first, into the flask. The glass tube attached to the cellophane sac and the base of the U-shaped glass tube were supported in a perpendicular position by cotton, forming the plug of the flask. A small glass tube was slipped over the free end of the stop-cock inserted in the flask and supported in this position by a cotton plug.

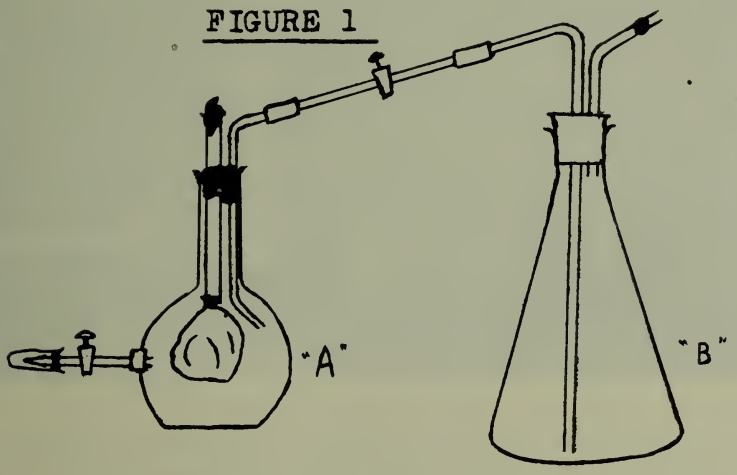
*Flask "B."*—A 1,000 c.c. erlenmeyer flask was marked off in 50 c.c. graduations with a diamond pencil and in it was placed 650 c.c. of synthetic medium prepared after the formula of Dorset, Henley & Moskey (2). A rubber stopper in which was a syphon and mouth piece, this latter plugged with cotton, was used as a plug for this flask.

By means of rubber joints, a straight glass stop-cock was connected at one end with the syphon of flask "B" and the other end with the longer arm of the U-shaped glass tube of flask "A." Parts of the apparatus where cotton was employed were covered with paper, the rubber stopper of the erlenmeyer flask loosened, the stop cocks closed and the assembled apparatus sterilized in the autoclave. In figure 1, a diagrammatic sketch of the apparatus is given.

At the same time, into three more florence flasks with cellophane sacs similar to flask "A," but lacking other modifications, 200 c.c. of synthetic medium, prepared at the same time and with the same ingredients as that in flask "B," were placed in each and contact between the cellophane sacs and fluid surfaces broken by raising the sacs. Three more florence flasks, without any modifications, received 200 c.c. of the same synthetic medium in each and were plugged with cotton. These six flasks were sterilized at the same time as flasks "A" and "B." After sterilization and cooling, the rubber stopper was replaced firmly in flask

" B " and bovine tubercle bacilli, strain No. 110, was planted in each of the seven florence flasks. The four with cellophane sacs were inoculated by placing a small flake of the tubercle bacilli, strain No. 110, inside each sac via its glass tube attachment and the three without sacs by floating a flake of the same culture on the surface of the medium in each flack. In case of flask " A," 200 c.c. of the medium from flask " B " were syphoned into it by opening the stop-cock connecting these two flasks and starting the syphon by blowing through the mouth piece, after which the stop-cock was closed and a short piece of rubber placed over the mouth piece and clamped with a pinch cock to prevent evaporation of the medium during incubation. The cellophane sacs, in all flasks so modified, were then brought in contact with the medium in them, by the aid of the glass tubes attached to them. To avoid undue evaporation of the media in the three flasks with cellophane sacs, prepared similar to flask " A," the cotton plugs were saturated with paraffin, but not the plugs in the glass tubes attached to the cellophane sacs. The flasks with the surface growths were not paraffined however.

FIGURE 1




At various periods after incubation, the fluids from one flask of each group of three flasks and from flask " A " were collected and made into tuberculin. In case of the diffusates from the flasks with the cellophane sacs, these were not heated before concentration nor were they filtered, while the culture filtrates from the three flasks with the surface growths were heated for one hour at 90° C. then passed through filter paper and a N Berkefeld filter. All samples were concentrated to one tenth of the original volume of media over a water bath, during which the temperature of the concentrated fluids was never higher than 78°C. All samples were preserved with 0·5 per cent phenol.

As a diffusate was withdrawn from flask " A," via the stop-cock, it was replaced by another 200 c.c. of medium from flask " B." By replacing the medium in flask " B " when exhausted with more synthetic medium, seven different diffusates were obtained from the original growth in the cellophane sac of flask " B," representing 1400 c.c. of synthetic medium and extending over a period of four hundred and eighty-eight days. The continuous diffusion of the products of bacterial growth was interrupted at this time, due to the disintegration of the cellophane sac and the last diffusate obtained and identified as tuberculin No. 93, was for a short time (not more than ten days in direct contact with part of the bacillary growth. The accompanying photograph was made during the fifteenth month of cultivation.

For the comparison and study of potency there were then the following tuberculins:—

*Group 1.* Represented by culture diffusates of different ages from a single culture growing in the cellophane sac in Flask "A," each diffusate corresponding to new media added during growth and comprising the tuberculins identified as Nos. 81, 84, 87, 90, 91, 92 and 93.

*Group 2.* Represented by the culture diffusates of increasing age from cultures in three different dialysing sacs, sustained by the original media on which they were cultivated, and comprising the tuberculins identified as Nos 82, 85 and 88.

*Group 3.* Represented by culture filtrates of increasing age from surface growths in three different flasks on the original medium and comprising the tuberculins Nos. 83, 86 and 89.

DETERMINATION OF POTENCY BY *in vivo* AND *in vitro* METHODS

Two comparative tests were made for the determination of potency at approximately one and one-half and two and one-half years after the first cultures were made. In the first comparative test tuberculins Nos. 81 to 90 inclusive were studied by the lethal method in tuberculous guinea pigs, by the intracutaneous method in four tuberculous horses and by the complement fixation method. In the second comparative study tuberculins Nos. 81 to 93 inclusive were studied by the lethal and intracutaneous methods in tuberculous guinea pigs and by the complement fixation method.

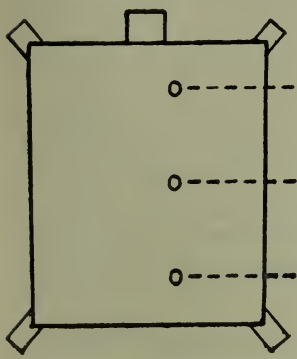
### *Lethal Dose Method*

In the first lethal dose test, guinea pigs inoculated six weeks previously by a recently isolated bovine strain, No. 206, were employed, and for the test received intra-abdominally, in groups of five, 2·5 c.c. of 1:10 dilution of each concentrated tuberculin, per 500 grammes live weight. In the second test, the same infective strain was used to sensitize the guinea pigs, but in this instance they were tested on the thirty-fifth day after infection.

### *Intracutaneous Method*

For the first intracutaneous test tuberculous horses were employed, which had been previously infected at periods ranging from one month to five years at the time the test was made. Different areas in the skin on the side of the

neck were utilized for the inoculation of the various tuberculins diluted 1:10 in 0·05 c.c. doses. In computing the results a rough average was struck and recorded by plus signs. In the second intracutaneous test, the tuberculous guinea pigs employed were from the same batch used for the lethal dose method in the second comparative test. A group of three guinea pigs were inoculated to determine the potency of two tuberculins, using as a standard for comparison a tuberculin of proven activity, No. 86, in every guinea pig of each group as indicated in the following diagram:—




The amount inoculated was 0·05 c.c. of 1:10 of the crude of each sample, and the potency of the various tuberculins were computed by totalling the degree of the reaction as estimated by signs + to ++++ of each tuberculin and comparing it with the total signs given by the same arbitrarily used as a standard.

*Complement Fixation Method*

In both the first and second tests, the usual complement fixation method of Watson & Heath (3) was used, the antiserum in each test being the same and identified as H83/Z1A, particulars of which will be found in study IV, and when employed was one week old in the first series of tests and over one year old in the second series.

In the following tables are given the results. In table 1 will be found the information pertinent to the preparation of the tuberculins, classified in groups according to the method by which the diffusates and culture filtrates were obtained and in tables 2 and 3, the methods of the first and second series of comparative tests respectively:—

TABLE 1

| Tuberculin | | Synthetic medium | | Days in contact with T.B. growth | | Dry Weight (in grammes) recovered from | |
|---|---|---|---|---|---|---|---|
| Group | Identity | Original volume in c.c. | Recovered volume in c.c. | Diffusates | Culture filtrates | Diffusates | Culture filtrates |
| 1..... | 81 | 200 | 130 | 90 | | | |
| | 84 | 200 | 115 | 60 | | | |
| | 87 | 200 | 135 | 56 | | | |
| | 90 | 200 | 100 | 67 | | | |
| | 91 | 200 | 125 | 69 | | | |
| | 92 | 200 | 148 | 109 | | | |
| | 93 | 200 | 165 | 37 | ..... | 8·1 | |
| 2..... | 82 | 200 | 165 | 90 | ..... | 1·7 | |
| | 85 | 200 | 155 | 150 | ..... | 1.5 | |
| | 88 | 200 | 140 | 206 | ..... | 1·35 | |
| 3..... | 83 | 200 | 125 | ..... | 90 | ..... | 1·68 |
| | 86 | 200 | 85 | ..... | 150 | ..... | 1·5 |
| | 89 | 200 | 65 | ..... | 206 | ..... | 1·5 |

TABLE 2.—FIRST COMPARATIVE TEST

| Identity of tuberculin | Complement fixation method | Lethal dose method | | | | | Intracutaneous method |
|---|---|---|---|---|---|---|---|
| | Value per 1 c.c. crude | No. g. pigs tested | Reactions at 24th hr. | | | | Average reactions in tuberculous horses |
| | | | Died | Strong | Weak | None | |
| 81 | 10,000 | 5 | 3 | – | – | 2 | +++ |
| 82 | 1,000 | 5 | 2 | 1 | – | 2 | + |
| 83 | 15,000 | 5 | 3 | – | – | 2 | +++ |
| 84 | 12,000 | 5 | 4 | – | – | 1 | +++ |
| 85 | 5,000 | 5 | 4 | – | – | 1 | +++ |
| 86 | 20,000 | 5 | 4 | 1 | – | – | ++++ |
| 87 | 8,000 | 5 | 3 | – | – | 2 | ++ |
| 88 | 3,500 | 5 | 5 | – | – | – | ++ |
| 89 | 15,000 | 5 | 3 | – | – | 2 | +++ |
| 90 | 15,000 | 5 | 5 | – | – | – | +++ |

TABLE 3.—SECOND COMPARATIVE TEST

| Identity of Tuberculin | Complement fixation method | Lethal dose method | | | | | Intracutaneous method | | |
|---|---|---|---|---|---|---|---|---|---|
| | Value per 1 c.c. crude | No. g. pigs tested | Reactions at 24th hr. | | | | Total reaction in 3 G.P. at 48th hr. | | Deviation from standard |
| | | | Died | Strong | Weak | None | Standard | Sample | |
| 81 | 10,000 | 5 | 5 | – | – | – | 9½ | 8 | −1½ |
| 82 | 500 | 5 | 1 | – | – | 4 | 9½ | 3½ | −6 |
| 83 | 12,000 | 5 | 3 | – | – | 2 | 9 | 8 | −1 |
| 84 | 6,000 | 5 | 4 | – | – | 1 | 9½ | 7 | −2½ |
| 85 | 2,500 | 5 | 3 | – | – | 2 | 11 | 8½ | −2½ |
| 86 | 20,000 | 5 | 4 | – | – | 1 | Employed as standard | | 0 |
| 87 | 2,000 | 5 | 3 | – | – | 1 | 9 | 5 | −4 |
| 88 | 2,000 | 5 | 3 | – | – | 2 | 9 | 6 | −3 |
| 89 | 6,000 | 5 | 5 | – | – | – | 7 | 4 | −3 |
| 90 | 10,000 | 5 | 4 | – | – | 1 | 6½ | 5 | −1½ |
| 91 | 12,000 | 5 | 3 | – | – | 2 | 5 | 4 | −1 |
| 92 | 10,000 | 5 | 3 | – | – | 2 | 6 | 5 | −1 |
| 93 | 3,000 | 5 | 2 | 1 | – | 2 | 10 | 7 | −3 |

## Comment

A cursory review of tables 2 and 3 will reveal so much conflict that any attempt at comparison between the *in vivo* and the *in vitro* methods or even between the two *in vivo* methods only in each test or between the two tests as a whole appears rather futile. For instance, tuberculin No. 88 in the first test with a value of 3,500 units killed five out of five guinea pigs tested by the lethal dose method and gave a two plus reaction in the intercutaneous test in horses, while tuberculin No. 83, with 15,000 units, killed only three out of five guinea pigs but gave a three plus reaction by the intracutaneous method. Comparing the results of the two tests, the conflict between the methods were only increased. In the complement fixation test for instance, only two tuberculins retained their original titres, viz., Nos. 81 and 86, all others having decreased by from one third to three quarters. In the lethal method also the variation was as great as only two of the tuberculins, Nos. 83 and 84, appearing in both tests gave identical results, while three have apparently increased in potency, viz., Nos. 81, 87 and 89 while five, Nos. 82, 85, 86, 88 and 90 have decreased The results by the intracutaneous method in the two tests do not permit of comparison, since the results were recorded differently in two classes of animals, but the

agreement appears closer between this method in the two tests than between the other methods.

The tuberculins in tables 2 and 3 were arranged in their numerical order of identity. By a rearrangement of them in groups according to the method of preparation as in table 1, a different light will be shed on the picture, as reference to tables 4 and 5 will indicate. The conditions under which tuberculins are prepared apparently have an important bearing on intracutaneous activity and the presence of detectable complement fixation bodies. In a repetition made of the methods of cultivation employed in this study with respect to the sealing of the cotton plugs in the mouths of the flasks in which cultures were growing, it was found that irrespective of whether the tuberculins were prepared from culture filtrates of surface growths or from the diffusates of growths in cellophane membranes, sealing the cotton plugs in the mouths of the flasks with paraffin, always caused a reduction in intracutaneous activity and impaired evaluations given by complement fixation titrations, as compared with control tuberculins prepared from the same strain, under identical conditions, except that the cotton plugs of the flasks from which these were prepared, were not sealed. This environment, however, apparently had no influence on the lethal activity of the tuberculins.

TABLE 4

| Tuberculin | | Complement fixation method | Lethal dose method | | | | | Intracutaneous method |
|---|---|---|---|---|---|---|---|---|
| Identity | Group | Value per 1c.c. crude | No. guinea pigs tested | Reactions at 24th hr. | | | | Average reactions in tuberculous horses |
| | | | | Died | Strong | Weak | None | |
| 81 | 1 | 10,000 | 5 | 3 | – | – | 2 | +++ |
| 84 | | 12,000 | 5 | 4 | – | – | 1 | +++ |
| 87 | | 8,000 | 5 | 3 | – | – | 2 | ++ |
| 90 | | 15,000 | 5 | 5 | – | – | – | +++ |
| 82 | 2 | 1,000 | 5 | 2 | 1 | – | 2 | + |
| 85 | | 5,000 | 5 | 4 | – | – | 1 | +++ |
| 88 | | 3,500 | 5 | 5 | – | – | – | ++ |
| 83 | 3 | 15,000 | 5 | 3 | – | – | 2 | +++ |
| 86 | | 20,000 | 5 | 4 | 1 | – | – | ++++ |
| 89 | | 15,000 | 5 | 3 | – | – | 2 | +++ |

TABLE 5

| Tuberculin | | Complement fixation method | Lethal dose method | | | | | Intracutaneous method | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Identity | Group | Value per 1 c.c. crude | No. guinea pigs tested | Reactions at 24th hr. | | | | Total reaction in 3 G.P. at 48th hr. | | Deviation from standard |
| | | | | Died | Strong | Weak | None | Standard | Sample | |
| 81 | 1 | 10,000 | 5 | 5 | – | – | – | $9\frac{1}{2}$ | 8 | $-1\frac{1}{2}$ |
| 84 | | 6,000 | 5 | 4 | – | – | 1 | $9\frac{1}{2}$ | 7 | $-2\frac{1}{2}$ |
| 87 | | 2,000 | 5 | 3 | – | 1 | 1 | 9 | 5 | −4 |
| 90 | | 10,000 | 5 | 4 | – | – | 1 | $6\frac{1}{2}$ | 5 | $-1\frac{1}{2}$ |
| 91 | | 12,000 | 5 | 3 | – | – | 2 | 5 | 4 | −1 |
| 92 | | 10,000 | 5 | 3 | – | – | 2 | 6 | 5 | −1 |
| 93 | | 3,000 | 5 | 2 | 1 | – | 2 | 10 | 7 | −3 |
| 82 | 2 | 500 | 5 | 1 | – | – | 4 | $9\frac{1}{2}$ | $3\frac{1}{2}$ | −6 |
| 85 | | 2,500 | 5 | 3 | – | – | 2 | 11 | $8\frac{1}{2}$ | $-2\frac{1}{2}$ |
| 88 | | 2,000 | 5 | 3 | – | – | 2 | 9 | 6 | −3 |
| 83 | 3 | 12,000 | 5 | 3 | – | – | 2 | 9 | 8 | −1 |
| 86 | | 20,000 | 5 | 4 | – | – | 1 | Employed as Standard | | 0 |
| 89 | | 6,000 | 5 | 5 | – | – | – | 7 | 4 | −3 |

The first comparative test, as given in table 4, reveals close agreement between the complement fixation method and the *in vivo* methods in groups 1 and 3. There was, however, some discrepancy between the complement fixation methods of the three tuberculins comprising group 2 and the lethal method, but agreement was fairly well maintained as between the complement fixation and intracutaneous methods, and on the whole there was a moderate degree of conformity between the complement fixation and the two *in vivo* methods for *the individual groups.*

In the second comparative test, table 5, the conformity is still maintained between the complement fixation and *in vivo* tests in *the individual groups,* and particularly so between the complement fixation and intracutaneous methods. A discrepancy between the complement fixation and lethal dose methods was found in group 3 of this test with tuberculin No. 89, which, with a titre of only 6,000 killed all five guinea pigs tested and actually appeared stronger than in the first test when the titre was 15,000, whereas in comparison tuberculin No. 83 of the same group, with double No. 89's complement fixation value, killed only three out of five guinea pigs by the lethal method but gave a much stronger reaction by the intracutaneous method than No. 89. It is obvious that some change had taken place in No. 89 during the interval between the first and second test and I have no better suggestion for explaining the change than that offered by Dorset, Henley & Moskey (2), namely, by hydrolysis.

A review of table 1 of the diffusates obtained continuously from the single culture, in conjunction with the potency of the corresponding tuberculins as given in tables 3 and 4, revealed that after growth was established at the ninetieth day, a period of sixty-seven days was necessary for the maximum production of potent tuberculins by this method, but no increased potency was obtained by a diffusate six weeks older, while diffusates sixty days and under did not attain full potency.

## Summary

1. Potent tuberculins were prepared from continuous diffusates of a single culture of bovine tubercle bacilli over a period of four hundred and eighty-eight days and the indications are that the only deterrent to indefinite production in this manner is the life of the diffusion membrane.

2. Tuberculins prepared from continuous diffusates, during the growth of a single culture of tubercle bacilli under the conditions described, are comparable in potency with tuberculins prepared from culture filtrates of surface growths, as indicated by *in vivo* and *in vitro* methods.

3. Tuberculins prepared by either method, however, are liable to undergo some undetermined change (probably hydrolysis) which diminishes both the intracutaneous activity and complement fixation value, but does not impair the lethal potency and may possibly enhance it (vide tuberculins Nos. 87 and 89 in the first and second tests, lethal dose method). Sealing the mouths of flasks in which cultures grew enhanced this change.

4. Diffusates produced in flasks sealed with paraffin and made into tuberculins (group 2) differ from tuberculins prepared from the diffusates produced continuously in an unsealed flask (group 1) in that those from the sealed flasks contained mainly the lethal principle with comparatively low content of the principle responsible for the skin and complement fixation reactions.

5. In the continuous diffusion products from a single culture of tubercle bacilli, a period of at least sixty-seven days contact with each replacement of new medium was apparently necessary, after growth was established, to obtain the maximum properties, but the potencies of the diffusates were not apparently enhanced to any extent by contact for longer periods. On the other

hand, a period of sixty days or less appeared to be too short to permit the possible maximum of tuberculin properties to diffuse in the sustaining medium.

6. Close agreement has been again demonstrated between the complement fixation and the intracutaneous methods in determining the potency of tuberculin.

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## VII. THE ACTIVITY OF TUBERCULIN FRACTIONS OBTAINED WITH ALCOHOL AS INDICATED BY *IN VIVO* AND *IN VITRO* TEST METHODS

Immediately following the appearance of the contributions of Mueller (1) on the fractionation of tuberculin by alcohol and his criticism of the complement fixation method as a means of standardizing tuberculin, preliminary studies were made with fractions of Old Tuberculin obtained by a similar technique to that employed by him since it was a matter of some importance to determine the accuracy of this Author's observations relative to the non-specificity of complement fixation reactions, for if these could be corroborated the method suggested by Watson & Heath (2) for determining potency and standardizing tuberculin would indeed be worthless.

### Technique

To 10 c.c. of O.T. prepared from culture filtrates of peptonated glycerinated bouillon media in the usual manner, 20 c.c. of 96 per cent alcohol were added, and after well mixing, centrifuged. The resulting precipitate was identified as " 2-volume precipitate." To the supernatant, 30 c.c. of 96 per cent alcohol were added and the precipitate after centrifuging identified as " 5-volume precipitate." The supernatant was identified as " 5-volume filtrate." The two precipitates were heated in the water bath at 65° C. for half an hour to drive off the alcohol and finally diluted to 10 c.c. The filtrate was evaporated until no odour of alcohol could be detected and distilled water added to make 10 c.c. Fractions by this same technique were obtained from concentrated peptonated glycerinated bouillon at the same time to serve as controls. The gross characteristics of the fractions obtained are given in table 1.

TABLE 1

| | Fraction | Colour | Other gross characteristics |
|---|---|---|---|
| Old Tuberculin | 2-vol. ppte..... | Greyish brown..... | Not entirely soluble in distilled water—precipitate greater than obtained with concentrated bouillon. |
| | 5-vol. ppte..... | Molasses..... | Sticky: readily soluble; foams excessively; precipitate greater than that obtained with concentrated bouillon. |
| | 5-vol. filtrate..... | Dark amber..... | Mainly glycerine after alcohol evaporated off. Only fraction with characteristic odour of tuberculin. |
| Concentrated peptonated glycerinated bouillon | 2-vol. ppte..... | Greyish sand..... | As corresponding ppte. from tuberculin but less in volume. |
| | 5-vol. ppte..... | Molasses..... | As corresponding precipitate from tuberculin but less in volume. |
| | 5-vol. filtrate..... | Light amber..... | As corresponding filtrate from tuberculin but without odour. |

The antigenic values of the six fractions were determined by the complement fixation method of Watson & Heath (2). As a check on the technique, the antigenic values of the unfractioned O.T., the unfractioned glycerinated peptonated bouillon and the mixed fractions from each were determined also. The results will be found in table 2.

It is of interest to note that the sum of the antigenic values of the three individual fractions of O.T. is less than the original unfractioned sample, but when mixed together and then titrated the antigenic value of the original is obtained. As noted in study IV this differs from fractions obtained by dialysis. Mixed fractions from all samples of tuberculin, do not, however, give the same results, as reference to table 6 of this study will reveal.

TABLE 2

| Product | Antigenic value per c.c |
|---|---|
| O.T. | 15,000 |
| 2-vol. ppte | 9,000 |
| 5-vol. ppte | 1,000 |
| 5-vol. filtrate | 500 |
| Mixed fractions | 15,000 |
| Concentrated peptonated glycerinated bouillon, unfractioned, fractioned or mixed fractions | Negative in dilutions 1:50 and over |

The *in vivo* activity of the O.T. and its various fractions were also determined by the lethal dose and intracutaneous methods, in guinea pigs infected thirty-nine days previously. Owing to an inadequate supply of test animals, however, the lethal dose method could only be carried out in a preliminary way, which was as follows:

The O.T. was diluted 1:4, and as each fraction may be qualitatively considered as one-third of the original sample these were diluted 3:4. The diluted preparations were administered intra-abdominally in doses varying from 2 c.c. to 0·25 c.c. per 250 grammes live weight of the animals inoculated. Tuberculous control guinea pigs received 2 c.c. of the various fractions of the concentrated peptonated glycerinated bouillon diluted 3:4, while healthy controls received similar doses of the fractions of O.T. The results will be found in table 3.

TABLE 3

| Guinea pigs | | Inocula | | | |
|---|---|---|---|---|---|
| No. | Weight in grammes | Identity | Per 250 gms live weight guinea pig | Antigenic value per 250 gms. live weight guinea pig | Result |
| 128 | 540 | O.T. diluted 1:4 | 2 c.c. | 7,500 | Dead in 6 hours |
| 126 | 500 | | 1 c.c. | 3,750 | " 5 " |
| 127 | 480 | | 0·5 c.c. | 1,875 | " 7 " |
| 139 | 440 | | 0·25 c.c. | 937 | " 18 " |
| 129 | 480 | 2-vol. ppte. diluted 3:4 (O.T.) | 2 c.c. | 13,000 | " 6 " |
| 130 | 460 | | 1 c.c. | 6,500 | " 7 " |
| 131 | 440 | | 0·5 c.c. | 3,250 | " 11 " |
| 135 | 480 | 5-vol. ppte. diluted 3:4 (O.T.) | 2 c.c. | 1,500 | " 7 " |
| 134 | 460 | | 1 c.c. | 750 | " 8 " |
| 132 | 440 | | 0·5 c.c. | 375 | " 8 " |
| 137 | 500 | 5-vol. filtrate diluted 3:4 (O.T.) | 1 c.c. | 375 | " 6 " |
| 138 | 440 | | 0·5 c.c. | 187 | " 26 " |
| 136 | 400 | | 0·25 c.c. | 93 | Sick—recovered |
| | | *Tuberculous Controls*— | | | |
| 143 | 480 | 2-vol. ppte. of glycer-pep- | 2 c.c. | Nil | |
| 144 | 480 | 5-vol. filtrate bouillon | 2 c.c. | Nil | |
| 145 | 400 | 5-vol. ppte. diluted 3:4 | 2 c.c. | Nil | |
| | | *Non Tuberculous Controls*— | | | Slight shock immediately after inoculation. All well 72 hours after. |
| 168 | 400 | 5-vol. filtrate of O.T. | 2 c.c. | 750 | |
| 169 | 400 | 2-vol. ppte. diluted | 2 c.c. | 13,000 | |
| 170 | 360 | 5-vol. ppte. 3:4 | 2 c.c. | 1,500 | |

For the intracutaneous tests three albino guinea pigs from the same batch used in the lethal dose method and two non-tuberculous pigs were employed for each sample. All preparations for inoculation were diluted 1:10 from the stock and administered in 0·05 c.c. doses at sites approximately one and a quarter inches apart in the shaven skin of the thoracic and abdominal walls, employing one side of the median line for the tuberculin and its fractions and the other for the concentrated peptonated glycerinated bouillon and its fractions. The results are given in table 4.

TABLE 4

| Preparation | Antigenic value per c.c. | Average reactions in | |
|---|---|---|---|
| | | 3 T.B. guinea pigs | 2 non T.B. guinea pigs |
| *Tuberculin*— | | | |
| O.T. diluted, 1 : 10 | 1,500 | +++ | - |
| 2-vol. ppte., 1 : 10 | 900 | ++ | - |
| 5-vol. filtrate, 1 : 10 | 50 | + | - |
| 5-vol. ppte., 1 : 10 | 100 | + | - |
| *Peptonated-glycerinated bouillon*— | | | |
| Concentrated bouillon, 1 : 10 | Nil | - | - |
| 2-vol. ppte., 1 : 10 | " | - | - |
| 5-vol. ppte., 1 : 10 | " | - | - |
| 5-vol. filtrate, 1 : 10 | " | - | - |

+ = Positive - = Negative ? = Questionable

The results recorded in tables 3 and 4 were the reverse of those obtained by Mueller. He discarded the "2-volume precipitate" as worthless, which by my results was shown to be the most active fraction, by all tests, while the "5-volume filtrate" which he considered the one most active was shown to be the least active. Moreover, there was a fairly definite relationship between the *in vivo* tests and the antigenic values obtained by the complement fixation method.

Mueller, however, had obtained the fraction he studied from concentrated tuberculin prepared from a synthetic culture filtrate, therefore, it was considered expedient to repeat this experiment with concentrated synthetic tuberculin before making any contrary statement, particularly as Abadjieff (3) had confirmed his results, using concentrated tuberculin, presumably prepared from culture filtrates of peptonated-glycerinated bouillon media, and also with a watery extract of tubercle bacilli. Abadjieff, unfortunately, did not consider information regarding the anti-serum and the technique employed in the complement fixation test, nor as regards the animals used for the skin tests, warranted, thus making a repetition of his work impossible.

In the prosecution of the proposed repetition of fractionation with alcohol, four tuberculins prepared on the synthetic medium of Dorset, Henley & Moskey (4) on which two different strains of tubercle bacilli had grown and two watery extracts from two different strains of tubercle bacilli, were selected and treated with 96 per cent alcohol, employing the same technique as previously given to obtain the "2-volume precipitate," the "5-volume precipitate" and the "5-volume filtrate" of each. The salient facts relative to the various tuberculins and watery extracts utilized for fractionation are as follows:—

*Tuberculin No. 72.* Prepared from a culture filtrate on which strain H37 (presumably the same strain utilized by Mueller for the production of tuberculins) had grown for one hundred and sixteen days on synthetic medium. The growth killed by heat at 90° C. and after passage through paper and an N Berkefeld filter, concentrated to one-tenth the original volume and 0·5 per cent phenol added. Two months after preparation, the antigenic value of this tuberculin was 1:10,000 (see study V); one year later the value had dropped to 4,000; two years later the value had further decreased to 2,500 and five years later at the time employed in this experiment the titre was only 1,500.

*Tuberculin No. 76.* Prepared from the products of growth of strain Vallée which had diffused through a cellophane membrane in contact with synthetic medium during one hundred and seventeen days, concentrated without filtration to one-tenth of the original volume of medium and 0·5 per cent phenol added.

*Tuberculin No. 77.* Prepared from a culture filtrate on which strain Vallee had grown for one hundred and seventeen days. The preparation of the concentrated product being the same as tuberculin No. 72.

*Tuberculin No. 78.* Prepared from the products of strain Vallée which had diffused through a cellophane membrane during sixty-two days after growth had been already established for one hundred and seventeen days. In other words, it was a tuberculin prepared from the same culture and in the same manner as tuberculin No. 76, but on new medium.

The antigenic values of tuberculins Nos. 76, 77 and 78 were 10,000, 12,500 and 20,000 two months after preparation. One year later 5,000, 5,000 and 10,000, and three years later at the time employed in this experiment was 3,000, 3,000 and 8,000.

*Watery Extract No. 72.*—The growth of H37 obtained from tuberculin No. 72, was extracted successively with ether and alcohol and finally with 200 c.c.

distilled water heated at 70° C. for two days. The organisms were separated by paper and an N Berkefeld filter, and 5 per cent glycerine added to the filtrate which was then concentrated to one tenth of the volume of distilled water employed for extraction. As a preservative, 0·5 per cent phenol was added. The antigenic value at the time of preparation was 2,000; while three years later at the time employed in this experiment it had decreased to 600.

*Watery Extract No. 78.*—The growth of strain Vallée in a cellophane sac which had produced tuberculins Nos. 76 and 78, was treated in the same manner as in the preparation of Watery Extract No. 72. The antigenic value at the time of preparation was 2,000, but two and a half years later at the time employed in this experiment was only 350 units.

The various fractions and the unfractioned tuberculins and watery extracts were tested for potency by the complement fixation and precipitin tests *in vitro* and by the intracutaneous method *in vivo*. The antiserum employed in the *in vitro* tests was obtained from a gelding seventeen years old and because of its unusual response to infection the salient facts of its history are given.

Seven years previously this gelding, No. 12, received intravenously during a period of six months 37 mgs. of the attenuated bovine strain of tubercle bacilli, No. 110, which although usually producing a high grade tuberculin did not stimulate anti-bodies in the serum of this animal suitable for the titration of tuberculin by complement fixation methods. The following year the animal received 20 mgs, of another strain of higher virulence, but again no suitable anti-serum was produced during a year, even after a strong response to the intracutaneous inoculation of tuberculin on one occasion as will be shown in Study X. In the following year, the animal received no further infection, but during the next four years, 52 mgs. of other strains of bovine tubercle bacilli of average virulence were inoculated intravenously, but which did not cause the production of suitable antisera. Intracutaneous tuberculin reactions during this period always remained weak. Ten months after the last infection, however, an immense reaction was obtained to the inoculation of tuberculin intracutaneously; and twelve days following which, an anti-serum was collected which, although showing an antibody content of only twenty units per c.c. with tuberculin as antigen, was found suitable for titrating tuberculin by complement fixation. This anti-serum was identified as H12/Z3V. Precipitin properties were also present in this anti-serum as determined by adding suitable doses of unheated serum to various dilutions of active tuberculins and incubating at 37·5° C. for twenty-four hours, but not in serum heated at 60° C. for half an hour. Abortin and synthetic medium only caused no precipitates with the serum whether heated or not, under the same test conditions.

### TECHNIQUE

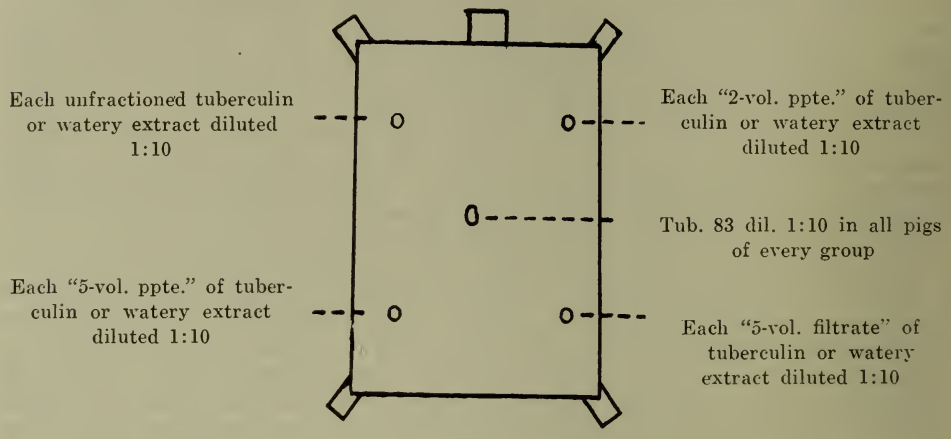
For the *complement fixation* method the usual technique of Watson & Heath (2) was employed, but it was found necessary to employ a dose of 0·2 c.c. of the anti-serum H12/Z3V.

For the *precipitin* method dilutions from 1:10 to 1:10,000 were made with the concentrated samples of tuberculins and watery extracts and their respective fractions, 0·25 c.c. of the unheated anti-serum H12/Z3V added to each dilution and incubated at 37·5° C. for twenty-four hours.

For the *intracutaneous* test, guinea pigs infected eighteen days previously with a bovine strain of moderate virulence, in a dose of 0·1 mgs. subcutaneously were utilized. Three white guinea pigs after removal of the hair from the thoracic and abdominal walls by shaving were used to test each tuberculin or watery extract and their respective fractions. As control, tuberculin No. 83 was employed in all pigs of every group. All the products were diluted 1:10 and inoculated in 0·05 c.c. doses. The reactions were recorded by the total

number of plus signs given by each sample in comparison to the control in each group of test animals. The tuberculin, No. 83, employed as a control for comparison revealed an antigenic value of 9,000 which was a decrease of 3,000 units since the last complement fixation titration one year before (see study VI).

The following diagrammatic figure will indicate the manner in which the intracutaneous inoculations were made in the various groups of animals:—




In the following table 5, are given the titres obtained by the complement fixation and by the precipitin methods of the various samples studied; also the reactions by intracutaneous inoculation, in the manner previously indicated, of the various inocula as compared with the reactions of the control tuberculin No. 83, in each group of three animals. The reactions were not so well marked as might have been desired and were undoubtedly due to the early date the test was made after the comparatively small infective dose. This test was purposely made at this time with a view to avoid having the animals too highly sensitive, since weak tuberculins will often give strong reactions in animals in a highly sensitive state out of proportion to their real value, making differentiation between moderately strong and weak tuberculins difficult.

## Comment

An examination of the results of the potency determinations as given in table 5 of the various fractions obtained from concentrated synthetic tuberculins with alcohol in comparison with those obtained with similar fractions from tuberculin prepared in peptonated glycerinated bouillon medium culture filtrates (O.T.) as given in tables 3 and 4, or even a comparison of the results of of the fractions of the concentrated synthetic tuberculins only, will reveal disagreement by the test methods employed, both individually and collectively, and the indications in table 5 are that varying degrees of antigenic properties *in vitro* and likewise, variations by intracutaneous activity *in vivo* may be expected in fractions obtained by similar treatment with alcohol from different samples of concentrated tuberculins when the samples utilized for fractionation are of different ages, prepared differently or obtained from different cultures. The fractions of the watery extracts studied revealed likewise a similar disregard for uniformity.

TABLE 5

| Identity of Samples | *In vitro* evaluation of samples before dilution per c.c. by | | Intracutaneous reactions at 48th hr. with | | Deviation from control |
|---|---|---|---|---|---|
| | Complement fixation | Precipitin | Control tuberculin No. 83 | Test samples (Col. 1) | |
| Tuberculin, 72 | 1,500 | 1,000 | 6 | 2 | −4 |
| 2-vol. ppte., 72 | 300 | 500 | 6 | 0 | −6 |
| 5-vol. ppte., 72 | 20 | 500 | 6 | 0 | −6 |
| 5-vol. fil., 72 | 100 | 100 | 6 | 0 | −6 |
| Tuberculin, 76 | 3,000 | 500 | 7 | 5 | −2 |
| 2-vol. ppte., 76 | 30 | 500 | 7 | 0 | −7 |
| 5-vol. ppte., 76 | 120 | 100 | 7 | 0 | −7 |
| 5-vol. fil., 76 | 600 | 10 | 7 | 1½ | −5½ |
| Tuberculin, 77 | 3,000 | 1,000 | 5½ | 2½ | −3 |
| 2-vol. ppte., 77 | 50 | 500 | 5½ | 0 | −5½ |
| 5-vol. ppte., 77 | 500 | 500 | 5½ | 0 | −5½ |
| 5-vol. fil., 77 | 600 | 500 | 5½ | 1 | −4½ |
| Tuberculin, 78 | 8,000 | 5,000 | 6 | 5 | −1 |
| 2-vol. ppte., 78 | 800 | 2,000 | 6 | 2 | −4 |
| 5-vol. ppte., 78 | 150 | 100 | 6 | 0 | −6 |
| 5-vol. fil., 78 | 2,000 | 500 | 6 | 2 | −4 |
| Watery Extract, 72 | 600 | 100 | 5½ | 0 | −5½ |
| 2-vol. ppte., 72 | 400 | 100 | 5½ | 0 | −5½ |
| 5-vol. ppte., 72 | 0 | 100 | 5½ | 0 | −5½ |
| 5-vol. filtrate, 72 | 150 | 100 | 5½ | 0 | −5½ |
| Watery Extract, 78 | 300 | 100 | 7 | 0 | −7 |
| 2-vol. ppte., 78 | 30 | 50 | 7 | 0 | −7 |
| 5-vol. ppte., 78 | 50 | 10 | 7 | 0 | −7 |
| 5-vol. filtrate, 78 | 200 | 100 | 7 | 0 | −7 |
| Control Tub. No. 83 | 9,000 | 5,000 | | | |

As indicated in table 2, the sum of the antigenic values as determined by complement fixation titration of individual fractions of O.T. prepared from peptonated glycerinated medium did not correspond to the original unfractioned sample, but did when mixed together and then titrated. This did not, however, hold true for all the fractions obtained from the four samples of concentrated synthetic tuberculins nor with the fractions obtained from either of the two watery extracts studied, as reference to table 6 will indicate, and in which will also be found the percentage of antigenic values retained by the individual fractions of one sample of O.T. and four of concentrated synthetic tuberculin as well as of the two watery extracts. The cause of the different results in this respect between mixed fractions of O.T. prepared from glycerinated peptonated bouillon medium culture filtrates and the mixed fractions of the concentrated synthetic tuberculins is not known, but was probably due to hydrolysis that had taken place in the course of fractionation with alcohol.

TABLE 6.—PERCENTAGE OF ANTIGENIC VALUES AS DETERMINED BY COMPLEMENT FIXATION TITRATION RETAINED BY INDIVIDUAL AND MIXED FRACTIONS OF TUBERCULINS AND WATERY EXTRACTS OF TUBERCLE BACILLI.

| Fractions | O.T. | Concentrated synthetic tuberculins | | | | Watery Extracts | |
|---|---|---|---|---|---|---|---|
| | | No. 72 | No. 76 | No. 77 | No. 78 | No. 72 | No. 78 |
| | % | % | % | % | % | % | % |
| 2-vol. ppte | 60·0 | 20·0 | 1 | 1·6 | 10·0 | 66·6 | 10·0 |
| 5-vol. ppte | 6·6 | 1·3 | 4 | 16·6 | 1·8 | 0·0 | 16·6 |
| 5-vol. filtrate | 3·3 | 6·6 | 20 | 20·0 | 25·0 | 25·0 | 66·0 |
| Mixed fractions | 100·0 | 66·6 | 100·0 | 100·0 | 75·0 | 50·0 | 50·0 |

Unlike the fractions obtained from O.T. prepared from the peptonated glycerinated bouillon culture medium, those obtained from three of the four

samples of concentrated synthetic tuberculin studied and as indicated in table 6, revealed that the greatest percentage of antigenic value was contained in the " 5-volume filtrate " and in this respect is in agreement with Mueller's findings. However, the fourth sample, No. 72 which was produced by the strain H37, revealed that the greatest antigenic value was in the " 2-volume precipitate " although the " 5-volume filtrate " contained approximately five times as many units as the "5-volume precipitate." Variations were also present in the fractions of the two watery extracts. However, irrespective of the variations among the different fractions of the synthetic tuberculins Nos. 72, 76, 77 and 78, no demonstrable skin reactions were obtained with any fraction with a titre under 600 by the complement fixation method, as table 5 will reveal, or in other words, approximately three antigenic units, as indicatd by the complement fixation test, were required to produce a reaction of one or one and one-half pluses in three tuberculous guinea pigs, in the particular state of hypersensitiveness they were in at the time tested. This, however, was not true with watery extract No. 72 which with a value of 600 or 3 units per 0·05 cc. of 1:10 dilution, the test dose, caused no reaction and was probably due to the low degree of sensitivity of the animals in this particular group as revealed by reaction given by the sample, No. 83, used for comparison.

A cursory compilation of the titres obtained by the complement fixation and by the precipitin tests of the unfractioned tuberculins as given in table 5, would indicate some rough relationship between these two test methods. The stronger tuberculins Nos. 83 and 78, with complement fixation titres of 9,000 and 8,000 respectively, had titres of 5,000 respectively by the precipitin test method; and tuberculins Nos. 72, 76 and 77 with complement fixation titres of 1,500, 3,000 and 3,000 respectively had titres of 1,000, 500 and 1,000 respectively by the precipitin test. This apparent relationship will be dissipated, however, if the titres of the various fractions are further compared by the two methods.

## Summary

1. Fractions obtained by similar treatment with alcohol from five samples of concentrated tuberculins prepared under different conditions do not reveal similar antigenic properties by complement fixation titration nor similar activity by intracutaneous test methods. Irregularities as regards antigenic properties by complement fixation titration were also present in the fractions of watery extracts of tubercle bacilli.

2. All of the individual fractions obtained by alcohol treatment from concentrated tuberculins contained lower antigenic values by complement fixation titration and less activity by intracutaneous tests than the original unfractioned samples and the fractions appeared to be unequal divisions of their original respective samples. Although without activity by intracutaneous test methods the fractions of the watery extracts studied, indicated similar behaviour, from the results of complement fixation titrations.

3. The complement fixation method of Watson & Heath (2), indicated fairly accurately the skin reacting activity of the fractions obtained by alcohol from the various tuberculins.

4. Under the experimental conditions of this Study, the precipitin test revealed no evidence of a specific relationship with the skin reacting principle of tuberculin, nor with the complement fixation test method.

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## VIII. THE EFFECT OF PROTEOLYTIC ENZYMES UPON THE ACTIVITY OF TUBERCULIN AS INDICATED BY *IN VIVO* AND *IN VITRO* TEST METHODS

As a method of approach to the study of the chemical nature of tuberculin many workers have made use of proteolytic enzymes. Recent investigations in this respect were those of Seibert (1), who reviewed the relative literature, which for the sake of brevity will not be repeated, and found that proteolytic enzymes, with the exception of erepsin, destroyed the skin reacting principle of tuberculin when employed in a suitable environment. The evidence she presented indicated fairly conclusively that the active principle was protein in nature or at least some substance closely associated with protein. The scope of this study, however, is not concerned with the chemical nature of tuberculin except in so far as its active principle is affected by proteolytic enzymes, as indicated *in vitro* by complement fixation and precipitin tests and *in vivo* by the intracutaneous and lethal tests in tuberculous animals.

In a preliminary study a composite sample of several concentrated synthetic tuberculins was employed to determine the effect of certain proteolytic enzymes on its active principle as indicated by complement fixation, precipitin, and intracutaneous tests. To this end the composite concentrated sample was diluted 1:10, filtered through paper, adjusted to $pH_7$ and divided into eight parts of 18 c.c. each. To the various samples, trypsin (Mercks) 5 per cent solution, pesin (Mercks) 2·5 per cent suspension, pancreatin (Lilly) 2·5 per cent suspension, normal hydrochloric acid and normal sodium hydroxide, were added in amounts as indicated in table 1.

The samples after treatment, along with an untreated sample to serve as control, were placed in the incubator for thirty-six hours, after which they were removed, the samples requiring it, neutralized and the volumes of each brought to 20 c.c. with distilled water. All samples were then placed in boiling water for half an hour.

TABLE 1

| Tuberculin sample | Trypsin 5% | Pepsin 2·5% | Pancreatin 2·5% | N/HC 1 | N/NaOH |
|---|---|---|---|---|---|
| 18 c.c. | 0·4 | – | – | – | – |
| 18 c.c. | 0·4 | – | – | – | 0·2 |
| 18 c.c. | – | – | – | – | 0·2 |
| 18 c.c. | – | 0·8 | – | – | – |
| 18 c.c. | – | 0·8 | – | 0·2 | – |
| 18 c.c. | – | – | – | 0·2 | – |
| 18 c.c. | – | – | 0·8 | – | – |
| 18 c.c. (Untreated Control). | – | – | – | – | – |

For the complement fixation determinations, the method of Watson & Heath (2) was employed, utilizing the same anti-serum (H12/Z3V) as in Study VII. For the precipitin determinations this same anti-serum was used but without inactivation, in 0·25 c.c. doses and which were added to various dilutions (1:10 in 1:1000) of the samples. Abortin gave no reactions with this serum in similar dilutions. For the intracutaneous test method a rabbit, injected intraperitoneally with an emulsion of a tuberculous lymph gland from a cow seven weeks previously, was utilized. The skin on the back of this animal, after the removal of the fur, by shaving, offered a suitable site for the comparative inoculations of the test samples in 0·05 c.c. doses. The results of these three methods are given in table 2.

TABLE 2

| Tuberculin sample treated with | Evaluation by | | Skin reactions |
|---|---|---|---|
| | Complement fixation | Precipitin | |
| Trypsin | 75 | 1,000 | ++ |
| Trypsin and N/NaOH | 25 | 1,000 | + |
| N/NaOH | 800 | 1,000 | ++++ |
| Pepsin | 250 | 1,000 | ++ |
| Pepsin and N/HCl | 25 | 1,000 | + |
| N/HCl | 500 | 1,000 | +++ |
| Pancreatin | 25 | 1,000 | + |
| Untreated control | 800 | 1,000 | ++++ |

+ = Positive reaction.

A review of table 2 indicates that trypsin and pepsin *per se* destroyed a portion of the active principle of tuberculin at neutrality, but a greater amount was destroyed by these enzymes in more appropriate environment of alkalinity and acidity respectively. Normal sodium hydroxide in the amount employed had no effect on the active principle while normal hydrochloric acid had to some extent. Pancreatin in the environment of neutrality destroyed the active principle as effectively as either trypsin in alkaline or pepsin in acid environments respectively.

As a result of these preliminary tests, samples of another synthetic tuberculin, No. 97, prepared from the filtrate of a surface growth of bovine strain, No. 110, were treated in the same manner as in table 1, with trypsin and normal sodium hydroxide, pepsin and normal hydrochloric acid and pancreatin, and along with an untreated control incubated for seventy-two hours, after which the samples requiring it were neutralized, the volumes made the same and all samples placed in boiling water for half an hour. The potencies of the various samples were then determined by *in vitro* and *in vivo* methods.

For the *in vitro* methods the complement fixation and precipitin tests were employed and executed in the same manner as in the preliminary tests, while for the *in vivo* methods, the intracutaneous and lethal tests were utilized. For the intracutaneous test three white guinea pigs, infected subcutaneously seven weeks previously with 0·1 mgs. of a bovine strain of tubercle bacilli, were employed and the four test samples without further dilution inoculated at four different sites in the skin of the abdominal and thoracic walls of each pig, after the hair had been removed by shaving. For the lethal method, each sample was inoculated into each of three tuberculous guinea pigs, from the same batch as served for the intracutaneous test, in a dose of 2·5 c.c. per 500 grammes live weight. The results of the four methods for determining potency will be found in table 3.

TABLE 3

| Tuberculin treated with | Evaluations by | | Lethal dose method | | | | | Intracutaneous test |
|---|---|---|---|---|---|---|---|---|
| | Complement fixation | Precipitin | No. guinea pigs tested | Results at 24th hr. | | | | Average reaction in 3 guinea pigs at 48th hr. |
| | | | | Died | Strong | Weak | None | |
| Trypsin and N/NaOH | – | 1,000 | 3 | – | – | – | 3 | – |
| Pepsin and N/HCl | – | 1,000 | 3 | 1 | – | 1 | 1 | – |
| Pancreatin | – | 1,000 | 3 | – | – | – | 3 | – |
| Untreated control | 500 | 1,000 | 3 | 3 | – | – | – | +++ |

## Comment

The results given in tables 2 and 3 further indicate the high degree of specificity given by the complement fixation method of Watson & Heath for titrating tuberculin, particularly in relation to intracutaneous activity and conversely indicate the non-specificity of the precipitin reactions and in this respect confirms the findings of Mueller (3). It is self-evident, moreover, that the principle responsible for the complement fixation and precipitin reactions are not identical and further supports the evidence presented in study VII in this respect.

Before any conclusive statement is warranted as regards the action of pepsin and hydrochloric acid and on the active principle of tuberculin more work is necessary, but the indications are that the combined actions of this enzyme and of hydrochloric acid appears to be directed more against the principle responsible for the intracutaneous and complement fixation reactions than that responsible for the lethal reaction, and may in part explain the reported discrepancies (1) obtained with this enzyme by different workers, depending on which *in vivo* method, the intracutaneous or lethal, was employed in determining the activity of treated samples. Moreover, commercial pepsins may vary considerably in their reducing action on tuberculin and one sample which was employed in an unreported experiment appeared to be either inert or incapable of acting on tuberculin. It is an established fact (4), however, that the behaviour of pepsin obtained from different animals vary with different proteins and to this may also be attributed its reported erratic action.

Hydrochloric acid *per se* even in the low concentration employed in this study appears to have some reducing action on the active principle of tuberculin, as indicated by the complement fixation and intracutaneous test methods employed (see table 2). The hydrolysing effect of this acid on the active principle of tuberculin will be further considered and will form the subject of study IX.

As a control on the results obtained with pancreatin, samples of the same tuberculin were treated with insulin, purchased on the open market, in volumes varying from one-tenth to five-tenths of the treated sample and incubated over a period of ninety-six hours. No loss in activity, as determined by the complement fixation method was found in any treated sample as compared with control samples under the same conditions, and the sample treated with the largest amount of insulin over the whole period of ninety-six hours was toxic for tuberculous guinea pigs to the same degree as the control, while non-toxic for healthy guinea pigs. Likewise positive intracutaneous reactions were obtained in tuberculous animals with this treated sample equal to the control, while healthy animals were negative.

## Summary

1. Under the experimental conditions stated trypsin and pancreatin completely destroyed the active principle of tuberculin as determined by complement fixation, intracutaneous and lethal dose test methods. Pepsin, on the other hand, in conjunction with hydrochloric acid did not destroy all activity and its reducing action appears to be directed more against the principle responsible for the skin and the complement fixation reactions than against the lethal principle.

2. The reducing action of the proteolytic enzymes employed, in whole or in part, on the active principle of tuberculin, had no effect on the precipitin titres.

3. Insulin under the conditions of the test had no reducing action on the active principles of tuberculin as indicated by complement fixation, intracutaneous and lethal dose methods.

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## IX. THE EFFECT OF ACID HYDROLYSIS UPON THE ACTIVITY OF TUBERCULIN AS INDICATED BY *IN VIVO* AND *IN VITRO* TEST METHODS

As shown in the previous study, No. VIII, the addition of a small amount of hydrochloric acid to tuberculin followed by heat and neutralization by soda destroyed a portion of its active principle as indicated by intracutaneous and complement fixation test methods which indicated agreement with the published results of Long (1), who found that the skin reaction principle of tuberculin could be totally destroyed if sufficiently hydrolysed by acid, but information regarding the effect of experimental hydrolysis on the lethal principle of tuberculin could not be found in the literature reviewed.

The method employed by Long (1) to produce hydrolysis appeared well adapted to further challenge the specificity of the complement fixation method for determining potency of tuberculin and also as a critical test method of the hypothesis advanced by Dorset, Henley & Moskey (2), with which the writer has expressed agreement (see study VI), to account for the principle found in certain tuberculins which is responsible for the specific lethal reaction as against the principle containing both skin reacting and lethal properties.

As a preliminary for orientation, several samples of concentrated synthetic tuberculins of various ages, prepared from different strains under dissimilar conditions were diluted 1:10 and adjusted to neutrality. To 10 c.c. samples of each, normal hydrochloric acid was added to make the final concentration of acid approximately n/100, n/20, n/10, n/6 and n/3 according to the technique of Long (1). The proteins precipitated by this treatment in the different samples with the concentrations of acid indicated, varied considerably in volume regardless of their intracutaneous activity, also in some samples no precipitation occurred with any concentration of acid even after twenty-four hours, which observation is at variance with the results given by Long.

It soon became evident, on examination for the cause of irregularities in acid precipitation, that tuberculins prepared from the bovine strains, Vallée and No. 110, gave somewhat smaller precipitates after the addition of acid than did a sample prepared from the human strain H37. The chief cause of variation, however, appeared to be the method employed for the production of the different samples, for in every instance, with one exception, where precipitates were absent after treatment with acid, the tuberculins were prepared from the diffusion products of growths of tubercle bacilli. The exception mentioned was tuberculin No. 80, which as indicated in study IV, although being from a culture filtrate of a surface growth, was prepared under unusual conditions, and had undergone a high degree of hydrolysis.

On the other hand, the addition of 20 per cent trichloracetic acid solution in 10 per cent amounts to tuberculin samples corresponding to those treated with normal hydrochloric acid, caused precipitates in varying degrees in all samples except No. 80. A slight precipitate, however, could be obtained from this sample with this reagent if added to saturation and allowed to stand overnight at room temperature. Likewise a similar precipitate as regards volume could also be

obtained in a previously untreated sample of No. 80, by the addition of ammonium sulphate to saturation followed by standing at room temperature overnight. The precipitates obtained with trichloracetic acid, unlike those obtained by normal hydrochloric acid, appeared to indicate a rough agreement between the amount of the precipitate and the activity of the tuberculin by intracutaneous tests and antigenic values in complement fixation titrations.

In table 1 will be found particulars of the tuberculins employed for precipitation as regards identity, strain utilized, age of culture when concentrated and whether prepared from culture filtrates of a surface growth or from the diffusion products of growth; also a rough indication of the amount of precipitation caused by the two precipitating agents employed, along with the indicated intracutaneous reactions and complement fixation titrations of dilute untreated samples.

TABLE 1

| Identity of tuberculin | Information relative to | | | Precipitates formed with | | Evaluations by | |
|---|---|---|---|---|---|---|---|
| | Strain | Age (in days) | Preparation | N/HCl (Maximum at any concentration) | 20% Trichloracetic acid in 10% amounts | Intracutaneous test | Complement fixation |
| 53 | H37 | 129 | Diffusate | – | trace | – | 15 |
| 54 | H37 | 129 | Filtrate | ++++ | ++ | + | 100 |
| 55 | No. 110 | 129 | Diffusate | – | +++ | +++ | 400 |
| 56 | No. 110 | 129 | Filtrate | +++ | + | + | 50 |
| 76 | Vallée | 117 | Diffusate | – | ++ | ++ | 300 |
| 77 | " | 177 | Filtrate | ++ | ++ | ++ | 300 |
| 79 | " | 182 | Diffusate | – | ++ | ++ | 300 |
| 80 | " | (250 (approx.) | Filtrate | – | – | – | – |
| 95 | No. 110 | 103 | Diffusate | – | +++ | +++ | 400 |
| 97 | No. 110 | 103 | Filtrate | +++ | +++ | +++ | 500 |

The results in table 1 indicated that the material precipitated by hydrochloric acid was not related to the skin reacting principle as demonstrated by tuberculins Nos. 53, 55, 76, 79 and 95, which although forming no precipitates with this reagent, still contained intracutaneous activity. This assumption could not be verified, however, for in experiments made to determine the activity of the precipitates and filtrates, obtained as a result of normal hydrochloric acid precipitation, by intracutaneous and complement fixation methods, in those samples forming precipitates, the active principle appeared to be fairly evenly divided between the precipitates and the filtrates, although, as just indicated samples giving no precipitates with normal hydrochloric acid were nevertheless active by these test methods. This may be illustrated as follows:—

A concentrated sample of tuberculin, No. 97, prepared from a culture filtrate of a surface growth on synthetic media, from bovine strain No. 110, was diluted 1:10, adjusted to neutrality and normal hydrochloric acid added to obtain maximum precipitation. The precipitate so formed was separated by centrifugal force, dissolved in an amount of normal sodium hydroxide equal to that of the normal hydrochloric acid employed for precipitation and brought to the same volume as the supernatant with distilled water. The supernatant was neutralized and with an untreated sample of the same tuberculin to serve as control, the activity of these three samples was determined by intracutaneous and complement fixation tests. At the same time another sample, No. 95, prepared from the same strain on similar medium for the same period of time but growing in a cellophane sac instead of directly on the surface of the medium, and which produced no precipitate when normal hydrochloric acid was added in any concentration up to n/3 was tested for activity in the same manner. The results will be found in table 2.

TABLE 2

| Tuberculin | Precipitate with N/HCl | Complement fixation titres | Intracutaneous reactions |
|---|---|---|---|
| No. 97 (surface growth) | +++ | 500 | ++++ |
| No. 97 (acid precipitate) | not applicable | 200 | ++ |
| No. 97 (supernatant) | not applicable | 250 | ++ |
| No. 95 (diffusate) | Nil | 400 | ++++ |

For the acid hydrolysis experiment the concentrated sample No. 97, appearing in tables 1 and 2, was employed and after diluting 1:10, filtering through paper and adjusting to neutrality, distributed in 8 c.c. amounts in seven test tubes. Normal hydrochloric acid was then added to the first five tubes in the following amounts:—0·08 c.c.; 0·4 c.c.; 0·8 c.c.; 2 c.c.; 4 c.c. to make the concentration of acid of each tube approximately n/100, n/20, n/10, n/6 and n/3 respectively. Precipitation was well marked with an accompanying loss in colour, immediately following the addition of acid in tubes 2, 3, 4, and 5. The precipitation and corresponding colour loss was slight in tube 1.

The five treated samples along with one of the two untreated were heated in the autoclave for half an hour at 120° C at fifteen pounds pressure. After heating it was noted that a change had taken place as regards the precipitates in certain tubes in that the precipitates in tubes 3, 4 and 5 had decreased while that in tube 1 had increased; that in tube 2, had apparently remained unchanged and the heated non-acidified sample, although quite transparent, was slightly darker in colour than the non-acidified unheated sample.

The five acidified samples were exactly neutralized with normal sodium hydroxide and six of the seven tubes brought to 16 c.c. volumes with distilled water to correspond with tube 5. The addition of the normal sodium hydroxide caused the precipitates to go into solution except in tubes 1 and 5 and in these traces of insoluble material remained. It was not considered necessary to make an exact adjustment of the sodium chloride concentration of the various samples.

For the determination of potency *in vitro* the complement fixation and precipitin tests were employed and for the *in vivo* determination, the intracutaneous and lethal tests were utilized in tuberculous guinea pigs. The techniques for these tests were as described in study VIII.

The results of this experiment as given in table 3, indicated that acid added to dilute synthetic tuberculin prepared in the conventional manner in a concentration of n/6 or higher and subjected to heat with pressure would destroy almost completely the skin reacting principle and the capacity to deviate complement while this severe treatment had little effect on the lethal principle.

TABLE 3

| Hydrolysed sample | Evaluation per c.c. by | | Lethal dose method | | | | | Intracutaneous method |
|---|---|---|---|---|---|---|---|---|
| | Complement fixation | Precipitin | No. guinea pigs tested | Reactions in 24 hr. | | | | Average reactions in 3 guinea pigs at 48th hr. |
| | | | | Died | Strong | Weak | None | |
| No. 1 x 0·08cc N/HCl | 200 | 500 | – | not tested | | | | +++ |
| No. 2 x 0·4c.c. " | 150 | 1,000 | 3 | 2 | – | – | 1 | +– |
| No. 3 x 0·8c.c. " | 75 | 1,000 | – | not tested | | | | – |
| No. 4 x 2 cc " * | 25 | 10 | 3 | 3 | – | – | – | trace |
| No. 5 x 4cc " | 15 | 10 | 3 | 2 | – | – | 1 | trace |
| No. 6 Untreated | 250 | 1,000 | 3 | 2 | – | – | 1 | ++– |
| No. 7 " no heat | 250 | 500 | 3 | 2 | | – | 1 | +++ |

* Samples 1-6 Heated in autoclave at 120°C—15 lbs. pressure.

Owing to the different reactions exhibited by tuberculins prepared from the diffusion products of tubercle bacilli growth, in comparison to tuberculins prepared from the culture filtrates of surface growths when normal hydrochloric acid was added (see table 1), it was considered desirable to hydrolyse at an acid concentration of n/3 a sample prepared by each method and determine their potencies comparatively.

To this end dilute samples of tuberculins No. 95 and 97 were employed, and as previously indicated, were prepared under similar conditions from the same strain except that the former was prepared by diffsusion while the latter in the conventional manner from the culture filtrate of a surface growth. To 8 c.c. of each diluted sample 4 c.c. of normal hydrochloric acid was added and both heated in the autoclave for half an hour at 120° C. and fifteen pounds pressure. After cooling, the samples were neutralized with 4 c.c. of normal sodium hydroxide. Potency determinations were then made by complement fixation, intracutaneous and lethal dose methods as previously indicated of the two hydrolysed samples along with an untreated sample of each of the two tuberculins, Nos. 95 and 97 suitably diluted to serve as controls, the results of which will be found in table 4.

TABLE 4

| Tuberculin sample | Complement fixation titre | Lethal dose method | | | | | Intracutaneous method |
|---|---|---|---|---|---|---|---|
| | | No. guinea pigs tested | Reactions at 24th hr. | | | | Average reaction in 3 guinea pigs at 48th hr. |
| | | | Died | Strong | Weak | None | |
| No. 97—hydrolysed at n/3..... | 15 | 3 | 2 | – | 1 | – | trace |
| No. 97—untreated..... | 250 | 3 | 1 | 1 | 1 | – | +++ |
| No. 95—hydrolysed at n/3..... | – | 3 | – | – | – | 3 | – |
| No. 95—untreated..... | 200 | 3 | 2 | – | – | 1 | +++ |

## Comment

A review of table 3 will show that the precipitin reactions were erratic and exhibited no specificity in comparison with either of the *in vivo* tests, although in the two higher concentrations of acid, the precipitins in the samples, were almost completely destroyed. On the other hand, the complement fixation reactions show a high degree of specificity in comparison with the intracutaneous test, both tests showing gradually decreased activity of the samples in ratio to the amount of hydrolysis present. There is, however, no agreement between the complement fixation titration and the lethal method or between the latter test and the intracutaneous test since the hydrolysing action of the acid, under the conditions of the experiment, had no destructive action on the lethal principle.

The lethal activity of the hydrolysed samples appear to further support the hypothesis advanced by Dorset, Henley and Moskey (2) concerning hydrolysis as the probable cause of the specific lethal principle found in tuberculin at the expense of the skin reacting principle, but cannot be accepted without reservations as an explanation of the manner in which hydrolysis actually occurs in tuberculin not subjected to experimental hydrolysis since it is inconceivable that free hydrochloric acid would ever be found in media, supporting cultures, in a concentration sufficient (the amount required experimentally being at least n/6) to cause the degree of hydrolysis capable of destroying the skin reacting principle. Moreover, tuberculin No. 80, which may be taken as an example of a tuberculin that had undergone hydrolysis (see study IV) without adventitious

aid, was found to have a neutral reaction after concentration and standing at room temperature for over three years, which would hardly be the case if the hypothetical hydrolysis had been due to hydrochloric acid.

In a review of the literature for any previous work that might shed light on the low pH concentration of tuberculin No. 80, assuming that hydrolysis had taken place in this sample, the work of Wherry and Ervin (3) appeared to offer a possible explanation. These authors found that $CO_2$ was a necessary requirement for the growth of tubercle bacilli and that this gas accumulated during growth. This finding, in conjunction with my own observations (see study VI) that tuberculins prepared in sealed flasks were invariably less active by intracutaneous and complement fixation test methods in comparison to lethal activity than corresponding tuberculins prepared in unsealed flasks, leads to the hypothesis that the $CO_2$ produced during growth of the organisms combines with the $H_2O$ of the media to form carbonic acid, which under certain peculiar conditions of environment, produces a concentration of acid in the culture medium suitable for hydrolysis. But the application of heat, usually employed in the preparation of tuberculin, causes the carbonic acid in the fluid medium of the culture to break down into its component parts of water and carbon dioxide and part of this latter is liberated, while a part combines with some other constituent of the media to form a carbonate, thus neutralizing the H ions and making it possible to record a low pH concentration in a sample hydrolysed without adventitious aid, such as tuberculin No. 80.

This hypothesis of acid hydrolysis by $H_2CO_2$ could not be supported experimentally, however, when $CO_2$ was bubbled through diluted synthetic tuberculin and then freed of the $CO_2$ by boiling, and the opportunity has not yet occurred to study the possibilities in this connection with unheated culture filtrates. The suggested change of the pH of tuberculin from neutral to acid following saturation with $CO_2$ on the other hand was found true, as was likewise its reversal to alkalinity after driving off $CO_2$ by boiling, in certain samples of tuberculin studied.

The results given in table 4, by the hydrolysed samples, Nos. 95 and 97, are somewhat contradictory as regards their *in vivo* reactions for in case of No. 95, the active principles—both skin and lethal—were completely destroyed by acid hydrolysis at a concentration of n/3, whereas No. 97, hydrolysed under the same conditions revealed destruction of only the skin reacting principle. A probable explanation of the result may be, as in case of precipitation with hydrochloric acid, due to the different methods utilized in preparing the two samples, but this phenomenon has not been sufficiently studied to warrant any definite statement.

## Summary

1. The addition of normal hydrochloric acid in concentrations varying from n/100 to n/3 did not cause precipitates in all samples of tuberculin capable of producing skin reactions in sensitive animals or similarly in those capable of deviating complement, although the acid precipitates *per se* from samples in which they were present, contained a part of the skin reacting principle as well as complement binding bodies of the corresponding unprecipitated samples.

2. Tuberculin samples capable of producing skin reactions in sensitized animals or of deviating complement produced precipitates in varying amounts after the addition of 20 per cent trichloracetic acid in 10 per cent amounts, and the volume of the precipitates appear to roughly correspond with the intensity of the skin reactions and the titres indicated by complement fixation.

3. Samples of a tuberculin prepared from the culture filtrates of a *surface growth* and hydrolysed with acid in concentrations varying from n/100 to n/3, revealed gradual loss of activity, as determined by skin tests and comple-

ment fixation titrations in proportion to the degree of hydrolysis, up to an acid concentration of n/10. An acid concentration of n/6 and greater practically destroyed all activity by these methods. On the other hand, hydrolysis within the limits of the experiment, that is to say, in concentrations up to and including n/3, had no apparent effect on its lethal activity. The precipitin reactions with hydrolysed samples although indicating loss of activity in the higher concentrations of acid were too erratic to permit of comparison.

4. A sample of tuberculin prepared from the *diffusion products* of growth of tubercle bacilli, lost all activity *in vivo* as determined by intracutaneous and lethal methods, and *in vitro* as indicated by complement fixation titrations when hydrolysed with acid at a concentration of n/3.

5. A hypothesis is advanced to explain the probable method in which hydrolysis of the active principle of tuberculin occurs ordinarily during growth.

6. The specificity of the complement fixation method of determining potency of tuberculin in relation to skin reacting activity is again indicated.

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## X. THE PRODUCTION OF ANTI-SERUM FOR TITRATING TUBERCULIN BY COMPLEMENT FIXATION TESTS

In the original contribution of Watson & Heath (1) on the standardization of tuberculin, it was stated that "No insurmountable difficulty need be anticipated in the production of this all-important reagent" (anti-serum) and they suggested that from further experimentation might result a method superior to the one they originally employed.

Studies were continued by the junior of the above authors over a period of more than six years in attempts to evolve a better and quicker method than that originally used. In the course of these various attempts, animals of both sexes comprising seventeen horses and two mules were employed. Fifteen of the former and the two latter received inoculations of seven different mammalian strains of tubercle bacilli, from various sources and of a wide degree of virulence (as determined by guinea pig infection), by intravenous and subcutaneous routes with live organisms of various ages and in various doses, grown on many varieties of media: also organisms killed by heat or chemicals and fractions of dead tubercle bacilli. In two instances inoculations of live organisms were supplemented by the injection of powdered sterile tapioca. The two remaining horses received avian tubercle bacilli isolated from a fowl and a hog respectively.

The technique employed for determining the antibody content of the antisera was as follows:—

Sera inactivated at 60°C. for half an hour were distributed in tubes in 1 c.c. amounts in ascending dilutions beginning at 1:5 and diluting as high as necessary to reach the end point. To each tube was added a predetermined dose of antigen, then titrated guinea pig complement was added in a 0·5 c.c. volume containing one unit. After incubation for one hour and ten minutes, washed 4 per cent red blood corpuscles of sheep and two units of rabbit anti-sheep haemolytic ambocepter were added, each in 0·5 c.c. volume and incubation continued for two more hours. The tubes after removal from the incubator were allowed to stand overnight at room temperature, and the degrees of fixation recorded by plus signs for positive, minus signs for negative, and plus-minus

signs for traces of red blood corpuscles below one plus. The positive reactions ranged from four plus (++++) indicating complete fixation to one plus (+) denoting only slight fixation of complement. The dilutions revealing three plus reactions, that is, the first one in which haemoglobin coloured the supernatant fluids above the sedimented corpuscles were arbitrarily selected as the titres of the anti-sera.

More than a thousand separate blood samples from horses and mules immunized under the varying conditions noted were tested by complement fixation methods as indicated, with two or more antigens, in an endeavour to determine if by such means, anti-sera suitable for determining potency of tuberculin could be differentiated from other anti-sera unsuitable, but giving positive reactions. In the earlier work concentrated tuberculin prepared from glycerinated bouillon and a methyl alcohol extract of tubercle bacilli were employed as antigens, and later two more antigens were added to these and were concentrated culture filtrates of tubercle bacilli grown on synthetic medium and a watery extract of tubercle bacilli. Incidently, it was found that the watery extract of tubercle bacilli obtained from dead tubercle bacilli after successive extractions with ether and alcohol, was by far the best antigen for diagnosing early infection by complement fixation methods and in combination with anti-sera usually revealed more antibodies than any of the other antigens, yet was itself low in antigenic units as compared with tuberculin.

Although serological titres in the same samples were often quite different with the four antigens, no useful information for identifying suitable anti-sera was available, besides the fact that one essential characteristic of a good antiserum was its ability to fix complement, not necessarily in high dilutions, with a tuberculin as antigen which elicited strong skin reactions in recently infected animals.

The intravenous route of infection gave better results than the subcutaneous and virulent bovine organisms were found superior to avirulent or attenuated ones for producing anti-sera, irrespective of the ability of these latter to produce tuberculin. At least one re-infective dose was always found necessary, and if adequate anti-sera were not obtained after two re-infections further inoculations at periods shorter than three months were usually futile. The inoculation of tapioca resulted in abscess formation, but unlike Ramon's (3) observation, relative to the production of diphtheria anti-toxin in horses, did not have the desired effect of stimulating antibodies demonstrable by the antigens employed, or in the production of suitable antiserum for determining potency of tuberculin. The two avian strains inoculated into two horses did not produce anti-sera for determining potency of even tuberculins prepared from the homologous strains and may be accounted for by the fact that the strains studied produced fulminating bacillemic disease (2).

There was also evidence to indicate that geldings were more suitable for the production of anti-sera than mares and mature animals—seven years or more—better than young animals; but even geldings meeting the age requirement did not always produce suitable anti-sera and in this respect was analogous to results obtained in the production of haemolytic amboceptors in rabbits, or, as commonly reported, in the production of anti-diphtheria toxin.

Observation of the results of complement fixation titrations of sera from experimentally infected horses and mules, at the time tuberculin was applied to them intracutaneously and at short periods thereafter, indicated that those animals which reacted positively exhibited an increase in antibodies following an initial decrease, examples of which are given in table 1.

The results obtained in table 1 suggested that this method might be utilized for producing suitable antisera for titrating tuberculin, so to this end, the same sera shown in table 1 was employed in 0·2 c.c. doses to determine the potency by complement fixation of a "standard" tuberculin, the results of which will be found in table 2.

TABLE 1

| Identity of infected animals | Intracutaneous tuberculin reactions | Serological reactions | | | |
|---|---|---|---|---|---|
| | | At tuberculinization with | 4 days later with | 8 days later with | 10 days later with |
| | | *Tub. 32 | * Tub. 32 | * Tub. 32 | * Tub. 32 |
| No. 7 | Negative | – | – | – | Not tested |
| No. 12 | Positive ++++ | 15 | 7½ | 25 | " |
| No. 18 | Positive + | 25 | 20 | 100 | " |
| No. 21 (Mule) | Positive ++++ | 50 | 25 | 100 | 80 |
| No. 26 | Positive + | – | – | 15 | Not tested |
| No. 31 | Positive ++++ | 10 | 5 | 200 | 200 |
| No. 44 | Positive ++++ | 20 | 5 | 80 | Not tested |

* Antigen. Tuberculin No. 32—prepared from culture filtrates of a bovine strain cultivated on synthetic media.
+ Degree of reaction. – = Negative.

TABLE 2

| Identity of animal supplying anti-serum | Antigenic values given a "standard" tuberculin | | | |
|---|---|---|---|---|
| | At tuberculinization | 4 days later | 8 days later | 10 days later |
| No. 7 | Nil | Nil | Nil | Not tested |
| No. 12 | Nil | Nil | 500 | " |
| No. 18 | Nil | Nil | 500 | " |
| No. 21 (Mule) | 500 | 500 | 8,000 | 10,000 |
| No. 26 | Nil | Nil | Nil | Not tested |
| No. 31 | 200 | 200 | 8,000 | 10,000 |
| No. 44 | Nil | Nil | 3,000 | Not tested |

As indicated in table 2, the anti-sera from Nos. 21 and 31, animals reacting positively, and obtained on the tenth day following the inoculation of tuberculin, each gave to the " standard " tuberculin a titre of 10,000 and appeared adequate for determining potency since the same evaluation was given this tuberculin when titrated originally with an anti-serum identified as H21/Z3N from No. 21, a mule gelding, collected twelve days after a re-infection with live tubercle bacilli and exhibiting an antibody titre at that time of 150 units per c.c.

Eighteen months had elapsed between the collections of anti-serum H21/Z3N and the sample collected from No. 21 at the time this animal was inoculated with tuberculin, during which period no inoculation of tubercle bacilli had been made. As shown in table 1, the antibody titre had dropped to 1:50 and four, eight and ten days after the inoculation of tuberculin intracutaneously the titres were 1:25, 1:100 and 1:80 respectively. As indicated in table 2, these various samples gave the " standard " tuberculin titres of 500, 500, and 8,000 and 10,000. The anti-serum from No. 31, a gelding, gave similar values to the " standard " tuberculin, on the eighth and tenth days after the inoculation of tuberculin, as anti-serum from No. 21, although their serum titres differed. On the other hand, anti-serum collected from No. 18, another gelding, on the eighth day after the inoculation of tuberculin, gave a value of only 500 to the tuberculin, although its titre was 1:100, and was no better for titrating tuberculin than serum from No. 12, with a titre of only 25. No. 44, a mare, indicated promise of suitable anti-serum, but the study of the serum from this animal was not continued. No. 26, although reacting positively to tuberculin applied intracutaneously and giving evidence of an increase of serum antibodies on the eighth day after inoculation, was without value to titrate tuberculin. The one remaining animal, No. 7, gave no reac-

tion to tuberculin applied intracutaneously and although infected exhibited no serum titres over the eight day period studied and like No. 26 was quite valueless to titrate tuberculin.

Following subsequent intracutaneous inoculations of several tuberculins fifteen months later, Mule No. 21 produced another supply of suitable anti-serum, identified as H21/Z5S, which was successfully employed to standardize tuberculins prepared from two different kinds of synthetic media (see study V). Attempts made in other tuberculous horses, however, to increase the titre of their anti-sera in an attempt to make them more suitable for use in determining potency, after the first inoculation of tuberculin intracutaneously, by repeated injections at short intervals (ten days), were unsuccessful, and in fact, diametrically opposite results were obtained. One or several intracutaneous inoculations of tuberculin into healthy horses did not cause the production of antibodies, however.

An increase in serum titres may also be obtained as a result of one subcutaneous inoculation of tuberculin into tuberculous horses, but not as a result of postiive ophthalmic reactions after the instillation of crude or precipitated tuberculin into the conjunctival sacs of their eyes. One animal inoculated with tuberculin by the intravenous route died in thirty hours.

All anti-sera produced as the result of the inoculation of tuberculin, like that sometimes produced by re-infection, were not always suitable for use in the complement fixation method for determining potency of tuberculin as indicated in table 2, and apparently their properties were dependent on the responses to the tuberculin inoculated at the time samples were collected, or in other words the state of the disease in the animals rather than on the kind or amount of tuberculin inoculated, for in some animals it was found to be only a matter of hours between suitable and unsuitable anti-sera. But anti-sera, produced in this manner, cannot, from the result of experimentation to date, be considered as anti-tuberculin serum, since its power to neutralize the activity of tuberculin is limited or absent, but the possibilities along this line of thought are still being investigated. Nevertheless, an anti-serum obtained as a result of inoculating tuberculin into a tuberculous animal and employed to determine the potency of tuberculin should escape Long's (4) criticism of "Complete dissociation of standardizing test and use to which the material is adapted." Even in the original contribution by Watson and Heath (1) this criticism was hardly warranted, since the anti-serum they employed for determinging tuberculin potency was obtained as a result of *reinfection*, which is, at least in part, analogous to the inoculation of tuberculin, and I would emphasize that a single infection, irrespective of the size of the dose, never produced in my hands, a suitable anti-serum.

Several adverse criticisms (4, 5, 6, 7, 8 and 9) have appeared in the literature with respect to the complement fixation method as offered by Watson and Heath (1) for the standardization of tuberculin, but in only two of them were the criticisms based on actual experimentation comparable with the original method, these being the contributions of Calmette and de Potter (5) and Aronson (6). In both these studies, the anti-sera prepared for the titration of tuberculins, were in many respects similar. In both, an antigen other than tuberculin was employed in determining the antibodies, although Calmette and de Potter also employed tuberculin. In both, avirulent or attenuated organisms were used as the inocula and both anti-sera revealed the presence of precipitins. The donor animals were, however, different, Calmette and de Potter employing a horse and Aronson a goat.

As shown in tables 1 and 2 of this study, there is apparently no relation between the titre of an anti-serum and its ability to determine the potency of tuberculin. Nevertheless, it seem unreasonable and undesirable to employ an antigen, such as an alcoholic extract of tubercle bacilli, to determine whether or

not an anti-serum would be suitable to titrate tuberculins for potency by complement fixation tests, for I have found that alcoholic extracts, freed from alcohol and incorporated into glycerine, have very poor and erratic tuberculin properties when inoculated into tuberculous cattle and guinea pigs intracutaneously, in comparison with ordinary tuberculin.

Much attention has been never given to precipitins, but in casual studies these properties have been found only in the sera of animals repeatedly inoculated with tubercle bacilli over a considerable period of time, but never in sera obtained within ten to fourten days after one or two re-infections over a short period (six weeks to two months), since precipitins appear at a much later date than do complement fixing bodies.

A horse* inoculated over a period of nine months with B.C.G. produced an anti-serum rich in precipitins for tuberculin but this same anti-serum was found incapable of titrating tuberculin by complement fixation. Moreover precipitins, produced with anti-sera in the presence of diluted tuberculins, appear to be non-specific, for as high titres have been obtained by me in precipitin titrations of tuberculin with serum obtained from an animal infected with *Trypanosome equiperdum* as with that from horses infected with tubercle bacilli.

Both complement fixing bodies and precipitins may, it is true, be present in the same sample of anti-serum, which is found suitable for determining tuberculin potency by complement fixation methods, as was shown in studies VII, VIII and IX, but they are by no means identical as Mueller (7) erroneously assumed and on which false premise his criticism of the complement fixation method was based, for precipitins found in anti-tuberculous horse or mule serum are destroyed at 60° C. for half an hour whereas such treatment has no effect on complement fixing bodies, and furthermore, they bear no relation to the skin reacting principles as was shown in the previous studies just referred to and may be further illustrated by the following:—

A tuberculin, No. 80, which by many tests was proven to be very weak or impotent, in so far as the skin reacting principle of tuberculin was concerned (see study IV), but almost superpotent by the lethal dose method, gave a positive reaction by the precipitin test in a dilution of 1:10,000, whereas it fixed complement at this time in no higher dilution than 1:75.

On the other hand, tuberculin No. 86, which was potent by both the skin and lethal dose tests and also fixed complement in a dilution of 1:20,000, gave exactly the same reaction in the precipitin test as tuberculin No. 80. However, when either tuberculin was combined with the anti-serium heated at 60°C. for half an hour, as employed in the complement fixation titrations, neither of them gave reactions in preciptin tests.

Reference to the article of Watson and Heath (1), and as indicated in this and previous studies, will show that there were some material differences between the methods employed in procuring anti-sera for utilization in determining potency of tuberculin by the complement fixing method by these authors and those employed by Calmette and de Potter (5) and by Aronson (6). Furthermore, the sample utilized by Calmette and de Potter deviated 60 units of complement in the presence of concentrated bouillon and on this count alone should never have been employed to determine the potency of tuberculin. Aronson (6) did not state whether the anti-serum from the goat which he employed revealed non-specific fixation reactions.

Fractions obtained by the ammonium sulphate precipitation from anti-tuberculous sera which had been found suitable for titrating tuberculin, were separated and also employed to determine potency of tuberculins with the hope of securing a higher degree of specificity. Only the pseudoglobulin fraction was found suit-

* This animal immunized at Animal Diseases Research Institute, Hull, and sample of serum obtained through the courtesy of Dr. E. A. Watson, Chief Animal Pathologist.

able for titrating tuberculin and the results obtained were comparable with the whole serum, but with a reduction in the antigenic values of approximately one-half those given by the unfractioned serum (see table 2—study III).

And so, up to the present time, no infallible method has been developed whereby suitable anti-serum can be obtained on every occasion. Nevertheless, if the following essential points are adhered to, suitable anti-serum may be obtained from most horses and mules:—

1. Healthy, vigorous horses or mules, at least seven years old, should be employed as donors, and geldings in preference to mares.
2. A fine suspension of a virulent strain of bovine tubercle bacilli inoculated intravenously. Exact dosage is not important, but should be large enough to establish infection and small enough to prevent a fulminating type of disease. Reinfection with an increased dose of finely suspended bacilli or the intracutaneous inoculation of one or more tuberculins should be made some time between the second and sixth weeks.
3. The collection of antiserum ten to fourteen days after a reinfection or after a strong reaction to the intracutaneous inoculation of one or more tuberculins. After the ninth day, test samples should be collected twice a day and tested at once. One essential of an adequate anti-serum will be its ability to fix complement in the presence of a suitably diluted dose of a tuberculin, which is capable of eliciting strong intracutaneous reactions in recently infected animals.
4. If the first or second reinfection or the first tuberculinization does not produce suitable anti-serum, inoculations should be discontinued for at least three months.

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## XI. THE NECESSITY OF AN OPTIMAL CONCENTRATION OF TUBERCULIN WHEN EMPLOYED AS ANTIGEN WITH HYPERIMMUNE ANTI-SERA IN COMPLEMENT FIXATION TESTS

During the early period in the study of methods for the production of anti-serum, an arbitrary antibody standard was adopted as a criterion for identifying suitable anti-serum for use in determining tuberculin potency. To meet the requirements of this standard, a minimum of 100 units per c.c. of anti-serum was considered necessary, when tuberculin as antigen was employed in a dose of one-half the full anticomplementary dose, which did not by itself cause any inhibition of the haemolytic system. It was soon discovered, however, that this arbitrary standard did not work out in practice, for anti-sera with as low as twenty units per c.c. was found suitable to standardize tuberculin, while others with as high as one thousand units per c.c. were unsuitable.

Theoretically the greatest quantity of antigen that can be utilized in the complement fixation method, which by itself causes no inhibition in the haemo-

lytic system, should be employed when the determination of maximum antibodies in a given anti-serum is desired. It is suggested that this holds true for bacterial antigens and for the watery and alcoholic extracts of microorganisms when employed as antigens with ordinary immune homologous anti-serum, but not for all products of bacterial growth of tubercle bacilli (tuberculins) and certain tuberculo-antigens (3) (4) when in combination with hyperimmune anti-sera and conversely when the value of these antigens are being determined by complement fixation tests against hyperimmune serum. Dean (1) has demonstrated a similar phenomenon in the case of anti-diphtheric serum when combined with diphtheria toxin, in complement fixation tests.

In support of the suggestion offered antigens of those types indicated were selected and studied against their respective *immune* anti-sera, in comparison with two tuberculo-antigens against a sample of tuberculous *hyperimmune* anti-serum. The antigens and anti-sera employed were as follows:

ANTIGENS

*Tuberculin No. 74.* Prepared by cultivating bovine tubercle bacilli, strain No. 110, on synthetic medium for 116 days, heating in the autoclave for one hour at 90°C. and then separating the growth by passage through paper and an N Berkefeld filter, concentrating to one-tenth the original volume and adding 0·5 per cent phenol as preservative.

*Watery Extract No. 32.* Bovine tubercle bacilli, strain No. 110, recovered from synthetic medium, washed with distilled water and after drying, extracted successively with ether and alcohol. The resulting residue then extracted in distilled water for forty-eight hours at 70°C. and finally heating for two hours at 95°C. The volume of the distilled water added for extraction was equal to the original volume of the medium from which the organisms recovered had been grown. The watery extract was finally passed through an N Berkefeld filter, 5 per cent glycerine added and concentrated to one-tenth of the original volume of distilled water used. As a preservative 0·5 phenol was added.

*"Abortin" No. 22.* Obtained by growing a virulent strain of *B. abortus* " Lennoxville " for one hundred and forty-five days on glycerine-dextrose-bouillon, separating the organisms by paper and an N Berkefeld filter, concentrating to one-tenth the original volume and 0·5 per cent phenol added.

*B. abortus No. 6730 suspension.* A forty-eight hours growth of this avirulent strain suspended in phenolized (0·5 per cent) normal saline (·85 per cent).

*Trypanosome antigen (2) No. 9, T. equiperdum*, being in the five hundred and fiftieth animal passage, collected from rats and suspended in 10 per cent glycerine to which was added 0·1 per cent formalin.

*Trypanosome alcohol-ether extract antigen No. 9a. T. equiperdum* as No. 9 collected from rats and extracted in equal parts of alcohol and ether.

ANTI-SERA

*Anti-tuberculous hyperimmune serum.* Obtained from a tuberculous mule infected with 72 mgs. of tubercle bacilli intravenously over a period of four years and collected following positive reactions to several tuberculins inoculated intracutaneously and identified as H21/Z5S.

*Anti-B. abortus serum.* Obtained from a cow artificially infected via the mouth with strain " Lennoxville " and identified as C35/J. The infected animal subsequently aborted.

*Anti-Trypanosome (Dourine) serum.* Obtained from a horse infected once intravenously with *T. equiperdum* and identified as H125/D.

The first step taken in the experiment was to determine the antigenic values of the various antigens against a standard amount of homologous anti-serum, utilizing for this purpose the technique of Watson and Heath (5), the results of which will be found in table 1.

TABLE 1

| Antigen | Serum employed | | Unit value of Antigen per c.c. |
|---|---|---|---|
| | Identity | Amount | |
| Tuberculin No. 74 | H21/Z5S | 0·1 c.c. | 30,000 |
| Watery Extract No. 32 | H21/Z5S | 0·1 c.c. | 2,000 |
| Abortin No. 22 | C35/J | 0·1 c.c. | 5,000 |
| *B. abortus* suspension No. 6730 | C35/J | 0·1 c.c. | 50 |
| Tryp. Antigen No. 9 | H125/D | 0·1 c.c. | 1,000 |
| Tryp. Alcohol-ether extract antigen No. 9a | H125/D | 0·1 c.c. | 500 |

With the antigenic values of the antigens determined, a series of dilutions were made of the three antisera and increasing amounts, in units, of their homologous antigens added to each. The results are given in table 2.

TABLE 2

| Anti-serum | Antigen employed | Titres given by anti-sera with following units of homologous antigens | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2½ | 5 | 10 | 20 | 25 | 35 | 50 | 100 | 350 |
| H21/Z5S | Tub. 74 | ..... | ..... | ..... | 80 | 60 | ..... | 25 | ..... | ..... | 10 |
| H21/Z5S | W. Extr. No. 32 | ..... | ..... | 50 | 75 | 80 | ..... | ..... | 1,000 | | |
| C35/J | Abortin | Neg. | ? | 800 | 2,000 | | | | | | |
| C35/J | *B. abortus* | ..... | 3,000 | 3,000 | 3,000 | | | | | | |
| H125/D | Tryp. susp. | ..... | 30 | 75 | 100 | 200 | | | | | |
| H125/D | Tryp. Alc. Extr. | ..... | 10 | 20 | 40 | ..... | 70 | .... | 100 | | |

Calmette and de Potter (6) in their titration of tuberculin by complement fixation employed the technique of Calmette and Massol (7), which although sound in theory, as it gives the actual number of competent units fixed, will be found rather burdensome in practice where a large number of titrations are to be made, due to the comparatively large amount of guinea pig complement that will be required. Also its predilection for demonstrating " Inhibitrice " in hyperimmune sera when in combination with certain tuberculo-antigens and with certain tuberculins makes it unsuitable for itratin gtuberculin generally for the determination of potency.

The technique of Calmette and Massol has not been extensively employed by me for the determination of tuberculin potency, but in a comparative study of twelve tuberculins by this technique and by that of Watson & Heath (5) comparable results were obtained, when the anti-serum employed was from an animal recently infected or, in other words, not " hyperimmune," as the results in table 3 will indicate.

On the other hand, if an anti-serum such as H21/Z5S, which may be considered as hyperimmune, it having been obtained from an animal after numerous inoculations of tubercle bacilli, is employed, the technique of Calmette & Massol, although admirable for demonstrating " Inhibitrice," will be found totally inadequate for titrating tuberculins for potency, if these happen to contain the principle which is capable of demonstrating the " Inhibitrice " phenomenon, as may be illustrated by the results as given in table 4.

TABLE 3

| Identity of Tuberculin | Values per c.c. of diluted tuberculin by | |
|---|---|---|
| | Technique of Calmette Massol | Technique of Watson & Heath |
| A | 0 | 0 |
| B | 400 | 600 |
| C | 100 | 150 |
| D | 300 | 350 |
| E | 0 | 30 |
| F | 50 | 60 |
| G | 125 | 200 |
| H | 50 | 60 |
| I | 200 | 350 |
| J | 200 | 250 |
| K | 400 | 800 |
| L | 300 | 300 |

TABLE 4

| Saline | Concentrated tuberculin No.74 dil. 1 : 50 | Anti-sera H21/Z5S dil 1: 5 | Complement units added | — | $\overline{aa}$ Haemolytic amboceptor and red blood corpuscles | Results |
|---|---|---|---|---|---|---|
| – | 1 c.c. | 0·5 c.c. | 1 | Incubated one hour ten minutes | 0·5 c.c. | Haemolysis |
| – | 1 c.c. | 0·5 c.c. | 5 | | 0·5 c.c. | " |
| – | 1 c.c. | 0·5 c.c. | 10 | | 0·5 c.c. | " |
| – | 1 c.c. | 0·5 c.c. | 25 | | 0·5 c.c. | " |
| – | 1 c.c. | 0·5 c.c. | 50 | | 0·5 c.c. | " |
| Controls | | | | | | |
| 0·5 c.c. | 1 c.c. | – | 1 | | 0·5 c.c. | Haemolysis |
| 1 c.c. | 1 c.c. | – | – | | 0·5 c.c. | No haemolysis |
| 1 c.c. | – | 0·5 c.c. | 1 | | 0·5 c.c. | Haemolysis |
| 1·5 c.c. | – | 0·5 c.c. | – | | 0·5 c.c. | No haemolysis |
| 0·5 c.c. | 1 c.c. | 0·5 c.c. | – | | 0·5 c.c. | " " |
| 1·5 c.c. | † | – | 1 | | 0·5 c.c. | Haemolysis |

## Comment

A study of table 2 will reveal that a certain similarity exists between the two bacterial filtrates, tuberculin No. 74 and abortin No. 22, in that they generally demonstrated less antibodies in their respective anti-sera than did the watery extracts of tubercle bacilli or the bacillary suspension of *B. abortus,* although as indicated in table 1, the antigenic values of the filtrates were much greater than those of the latter two antigens. The similarity between the two filtrates in this study went no further however, for when tuberculin No. 74 was utilized as an antigen to demonstrate maximum antibodies in accordance with the theory expressed it reacted reversely to abortin No. 22, as well as to the other four antigens since the greater the amount or number of units of tuberculin employed as antigen, the less were antibodies revealed, and supports the hypothesis advanced with regards the behaviour of tuberculin-*hyperimmune* anti-tuberculous serum mixtures in complement fixation as against other antigens and their *immune* anti-sera.

It is conceivable, however, that some of the other four antigens would have exhibited the same phenomenon as tuberculin No. 74 in demonstrating anti-

bodies had *myperimmune* instead of *immune* anti-sera been employed, for occasionally, I have observed this phenomenon with even certain bacillary suspensions antigens, but it is more often encountered and in a greater degree in bacterial filtrates than in other antigens.

The indications are that this phenomenon is identical or similar to the "Inhibitrice" of Calmette (3) or the Inhibitive Fixation of Caulfield (4) and like these, the two requisites for its demonstration are *hyperimmune* anti-serum and an antigen or antigens containing certain specific properties. It therefore follows that all *hyperimmune* anti-tuberculous sera will not necessarily exhibit the phenomenon with every tuberculo antigen, or with every sample of tuberculin and similar results may be expected when antigenic values are being determined, but in the determination of these latter, particularly by the technique of Calmette & Massol, there is the likelihood that misleading results will ensue, as the specific properties responsible for the consummation of inhibition of fixation vary in different samples.

As illustrated in table 3, the evaluations of tuberculins by the technique of Calmette & Massol (7) and that of Watson & Heath (5) respectively, did not vary to any marked degree, when an *immune* anti-tuberculous serum was employed, but on the contrary when *hyperimmune* anti-serum, such as the sample H21/Z5S, was employed to evaluate the antigenic properties of tuberculin No. 74 by these techniques, that of Calmette & Massol, as shown in table 4, indicated that this tuberculin was valueless, whereas as shown in table 1, the antigenic evaluation of this tuberculin by the technique of Watson & Heath was 30,000 units per c.c. of the concentrated product.

## Summary

1. The unit value of an antigen is not always an indication of its ability to demonstrate antibodies in anti-sera, particularly antigens prepared from culture filtrates of bacterial growth.

2. A sample of tuberculin employed as an antigen in complement fixation tests for determining maximum antibodies in a sample of *hyperimmune* anti-tuberculous serum did not conform to the rule applicable to a watery extract of tubercle bacilli as antigen with the same anti-serum or to that governing antigens of other micro-organisms when in combination with their *immune* homologous anti-sera.

3. The complement fixation method of Calmette & Massol is unsuited for the determination of tuberculin potency when a *hyperimmune* anti-tuberculous serum forms part of the antigen-antibody mixture.

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## XII. FACTORS GOVERNING SKIN NECROSIS FOLLOWING TUBERCULIN INJECTIONS

Macroscopic skin necrosis of tuberculous guinea pigs at the sites of intracutaneous inoculations of tuberculin has been stressed by many observers, whether their objective was the determination of the potency of a tuberculin fraction or hypersensitiveness produced by a particular strain of tubercle bacilli.

From observations over a period of years, I am inclined to the opinion that no great importance need be attached to the phenomenon of macroscopic skin necrosis in tuberculous guinea pigs when assessing the potency of a tuberculin by the intracutaneous test, because the technique employed in making inoculations for this test appears to be closely associated with the phenomenon, provided, of course, the guinea pigs tested are sensitive and the tuberculin employed is of adequate potency.

To challenge the validity of the opinion expressed, tuberculous guinea pigs, infected at various periods with different strains of tubercle bacilli, were selected and inoculated at two sites in the shaven skins of their abdominal walls, with the same sample of tuberculin in identical dosage. One inoculation was made superficially, while the other was made deeper. Two samples, Nos. 48 and 74, of concentrated synthetic tuberculins, suitably diluted, were utilized.

The duplicate inoculations of both samples of tuberculin employed caused distentions in the skins of the test animals which were plainly visible after withdrawal of the needle; but the former (superficial) resembled small water blisters, while the latter (deep) were more in the nature of thickenings.

To obviate the possibility of one part of the abdominal wall utilized being more sensitive than the other, the two methods of inoculating the tuberculins were alternated in the animals receiving the different samples, so that in one the superficial inoculation was proximal to the head while in the next animal it was distal.

Particulars regarding infection, texture of the skin, site utilized for different inoculations, identity and dose of tuberculins employed and resulting reactions will be found in table 1. Observations of the reactions were made from the 24th to the 96th hours after inoculating the tuberculin, but for convenience only those at the 48th hour, which are representative, are recorded in table 1.

### Comment

It is, I believe, a generally accepted fact that tuberculin of adequate potency, has a necrotizing action on the skin of sensitized tuberculous animals when applied intracutaneously. This may be taken for granted since it acts as an irritant in such animals, causing acute inflammation with resulting tissue injury, and when located at one point for any length of time, necrosis of single cells or groups of cells follows. This may be demonstrated histologically. (Table 1.)

For the demonstration of macroscopic skin necrosis, however, in conjunction with the other phenomena of acute inflammation, a great deal depends on the location of the tuberculin applied. If it be close to the surface macroscopic skin necrosis will ensue, usually followed by sloughing and ulceration. If on the other hand it be deep, macroscopic evidence of necrosis may not accompany the other phenomena of acute inflammation.

It is not quite clear if workers employing the intracutaneous test to determine the potency of tuberculin have fully appreciated this factor in technique and its influence on the presence of macroscopic skin necrosis at the point of intracutaneous tuberculin reaction.

TABLE 1

| Guinea pig No. | History of Infection | | | | Texture of skin | Innoculation sites utilized in relation to head | | Identity and dose of tuberculin employed | Reaction at 48th hour | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Route | Dose | Strain | Days infected | | Superficial | Deep | | Superficial | Deep |
| 804..... | S | 1 mg.. | No. 100 | 104 | Thick.... | P | D | ..... | +++VN | +++ |
| 805..... | S | 1 mg.. | No. 100 | 104 | " .... | D | P | ..... | +++VN | +++ |
| 907..... | S | 0·1 mg.. | Vallée.. | 40 | Medium.. | P | D | No. 48 0·005 c.c. diluted 1 : 5 | ++++VN | ++++VN |
| 908..... | S | 0·1 mg.. | " .. | 40 | Thick.... | D | P | | +++ | +++ |
| 909..... | S | 0·1 mg. | " .. | 40 | Thin..... | P | D | ..... | ++VN | ++ |
| 394..... | S | 0·1 mg. | No. 205 | 25 | Medium.. | D | P | ..... | ++++VN | ++++VN |
| 384..... | S | 0·05 cc. lymph gland | No. 206 | 31 | Medium.. | P | D | No. 74 0·05 cc. diluted 1 : 5 | ++++VN | ++++ |
| 385..... | I | | " .. | 31 | Medium.. | D | P | | +++VN | +++ |
| A..... | S | 0·01 mg | Vallée.. | 198 | Thick.... | P | D | | Died in 12 hours. | |
| B..... | S | 0·01 mg | " .. | 198 | " .... | D | P | | Died in 12 hours. | |

S=Subcutaneously
I=Intraperitoneally
P=Proximal

*=Blood exuded from needle puncture
VN=Visible necrosis
D=Distal

The texture of the skin of animals of different species or even individuals of the same species may be also a contributing factor in this phenomenon, when it is considered that macroscopic skin necrosis as a result of the intracutaneous inoculation of tuberculin into the caudal folds of cattle, is comparatively rare under ordinary field conditions. Likewise, my experience with experimental tuberculous horses and mules, which had received numerous and repeated inoculations of tuberculin intracutaneously in their necks, would indicate that typical macroscopic skin necrosis is a rarity in these animals, even when reactions recorded have measured as much as five inches in diameter. Macroscopic necrosis in the wattles of tuberculous barnyard fowls is also rare under ordinary conditions of applying the diagnostic test with avian tuberculin.

In so far as demonstrating macroscopic skin necrosis, resulting from the intracutaneous inoculation of tuberculin, in tuberculous guinea pigs, a review of table 1 will indicate that this phenomenon can be attributed to neither the infective strain, the period after infection, the tuberculins employed, the particular site utilized, nor, in this instance, to the texture of their skins. The resulting reactions obtained, however, associated the presence or absence of macroscopic skin necrosis with the technique practised in applying the tuberculin. The evidence in support of this being that the deep inoculations made at eight sites, two only revealed macroscopic necrosis and in each instance blood had exuded after withdrawal of the needle; while the superficial inoculations made at eight sites also, in the same animals, in similar dosage with corresponding samples of tuberculin, resulted in macroscopic necrosis at seven of the eight inoculated sites.

Although the infective strain of tubercle bacilli, the period after infection, that is to say, the state of the disease, and the tuberculin employed are all factors in the phenomenon of skin necrosis, both microscopic and macroscopic, the evidence presented would indicate that the technique practised when inoculating tuberculin intracutaneously in tuberculous guinea pigs has an important bearing in determining whether or not macroscopic skin necrosis will be associated with the other phenomena of inflammation present in a positive intracutaneous tuberculin reaction.

It therefore follows that if macroscopic skin necrosis is taken as a criterion of high tuberculin potency, errors will likely occur when assessing potency by the results of intracutaneous methods, even if due regard is given to the technique for making inoculations, as under ordinary conditions this cannot be rigidly controlled, and the errors will be comparatively greater if this factor is overlooked.

The deaths of guinea pigs "A" and "B," table 1, did not particularly indicate the high potency of tuberculin No. 74, but did reveal the high state of lethal sensitiveness present in these animals at the time the tuberculin was inoculated.


### Summary

1. Guinea pigs infected with four different strains of tubercle bacilli at periods ranging from twenty-five to one hundred and four days before being tested intracutaneously with two tuberculins, each in duplicate, revealed macroscopic skin necrosis in seven of eight inoculations made superficially, while with deeper inoculation only two of such inoculations revealed similar phenomena, and in each instance there was a rupture of small blood vessels in the skins following inoculation.
2. Alternating the inoculations with regard to the sites utilized did not appear to influence the appearance of skin necrosis; neither was there evidence that one reaction influenced the other in the same animal.
3. Two guinea pigs infected one hundred and ninety-eight days died as a result of the intracutaneous inoculation of tuberculin.


## RECAPITULATION

The outstanding feature of the evidence presented in the preceding experimental studies is the remarkable constancy of agreement between the complement fixation titrations and the results of intracutaneous inoculations in tuberculous animals of the tuberculins studied, which, as indicated, were obtained from different strains of tubercle bacilli, and in many instances were prepared under dissimilar conditions. This accord between the two test methods was further emphasized when it was still maintained with samples subjected to fractionation with alcohol or acid, reduction by proteolytic enzymes or hydrolysis by acid, and supports the published results of Dienies, Shoenheit and Scheff (1), who remarked on the parallelism between these two test methods, in their studies of certain fractions of tubercle bacilli.

The agreement between the complement fixation and lethal methods was not so constant and the irregularities appear to be dependent on the amount of hydrolysis present in the samples tested, for as was first suggested by Dorset, Henley and Moskey (2), and as indicated in some of the preceding studies, a principle with only specific lethal activity may be split off by hydrolysis from the original active molecule containing both skin reacting and lethal activities, so that in a single sample of tuberculin two specific principles may be present, one giving the intracutaneous-lethal reactions and the other responsible for the specific lethal reaction only. The manner in which the theoretical hydrolysis occurs during the preparation of tuberculin is not definitely known, but observations in this respect indicated that carbonic acid gas might be a contributing cause. Attempts, however, to support this hypothesis experimentally, feasible though it appeared, proved unsuccessful.

The former principle, responsible for both types of *in vivo* reactions in tuberculous guinea pigs, appears to be nondialysable and contains antigenic properties by complement fixation methods, while the latter, responsible for specific lethal effects only, is dialysable and lacks complement deviating properties. The exact separation of the two principles by dialysis, however, may not be always attain-

able for, as Long (3) has so aptly illustrated, molecules of all sizes appear to be the usual occurrence in a hydrolysed sample of tuberculin and the only possibility of accurate fractionation by this technique would be by means of specially prepared and graded dialysing membranes.

Dialysis of tuberculin and diffusion of tuberculin properties of tubercle bacilli during growth appear to be distinct phenomena, however, since active tuberculins containing the theoretical larger complex molecule which is responsible for the intracutaneous-lethal and complement fixation reactions, may be prepared from the diffusion products of tubercle bacilli during growth; while as shown in study III dialysis of tuberculin prepared from diffusates, whether heated or unheated, will permit only split molecules, or in other words, molecules responsible for the specific lethal reaction of those samples in which hydrolysis is present, to dialyse. The dialysis of concentrated tuberculins prepared in the usual manner gave similar results in three of four samples dialysed.

Sectioning, suitable staining and microscopic examination of the cellophane membrane employed to separate the growth of tubercle bacilli from the sustaining medium during production of tuberculin by diffusion did not support the hypothesis offered by a colleague (4) that the explanation of the phenomenon noted between dialysis and diffusion may be due to the partial penetration of the cellophane membrane by the organisms and in this way serve as distributing channels in absorbing nourishment from the media on the one hand and discharging the result of metabolism (tuberculin) into the medium on the other.

In passing, it is of interest to note that although tuberculins prepared by diffusion are indistinguishable from tuberculins prepared in the conventional manner from filtrates of surface growths, in so far as activity, as indicated by *in vivo* and by complement fixation methods, is concerned, the molecular structure of their active principles does not appear to be identical; for no precipitates could be obtained from samples of tuberculins prepared by diffusion when subjected to treatment with normal hydrochloric acid in concentrations from approximately n/100 to n/3 and when a sample of tuberculin prepared in this manner was hydrolysed at an acid concentration of n/3 in conjunction with heat and pressure, the active principle, as determined by intracutaneous, lethal and complement fixation methods, was completely destroyed. On the other hand, tuberculins prepared in the conventional manner, when similarly treated with normal hydrochloric acid, formed precipitates, and when hydrolysed in a corresponding manner to the tuberculin obtained by diffusion, only the principle responsible for the intracutaneous and complement fixation reactions was destroyed leaving the lethal principle unimpaired.

That active tuberculins, indistinguishable in so far as diagnostic reactions were concerned from those prepared in the conventional manner from culture filtrates of surface growths, could be obtained continuously from a single culture enclosed in a cellophane sac, was fully demonstrated and the only deterrent to indefinite production in this manner appears to be the life of the diffusion membrane. Although this method has no great advantages over the conventional one, as regards the production of diagnostic tuberculins, it opens up another method of approach for the study of the metabolic products from a single culture over an extended period.

The precipitin test under the conditions stated, unlike the complement fixation test, gave erratic and unreliable results when compared with either the intracutaneous or lethal methods, and the evidence obtained indicated that the reactions by this test method were not specific for determining tuberculin potency. It was also demonstrated that the principle in anti-sera responsible for the precipitin reactions was quite distinct from that causing complement fixation reactions, although both principles may be present in the same sample.

The fractionation of tuberculin with alcohol or with normal hydrochloric acid revealed that similar fractions could not be obtained from all active samples

similarly treated, when the samples studied originated from different strains of tubercle bacilli or were prepared under dissimilar conditions; and in conjunction with vagaries indicated in individual samples, it is obviously fallacious to attempt the formulation of general principles to govern the behaviour of all tuberculins when subjected to fractionation with these reagents from the results of experimentation on only a few samples and much less so from the study of a single one. Precipitation of dilute tuberculins by trichloracetic acid, however, regardless of their origin or method of preparation, indicated a definite relationship between the amount of precipitates obtained and the indicated activity of the samples by intracutaneous and complement fixation methods. A similar observation was made by Seibert (5) relative to the former test and she suggested the use of trichloracetic for standardizing tuberculin.

As regards proteolytic enzymes: The destruction of the active principle of tuberculin by pancreatin might suggest that its action was due to the amylopsin present in this complex enzyme and indicate that the active principle of tuberculin is associated with a carbohydrate. This hypothesis, however, is untenable since the active principle of tuberculin was destroyed by trypsin *per se*, a proteolytic enzyme also present in pancreatin, while insulin *per se*, a pancreatic hormone, had not the slightest effect. The active principle of tuberculin under the conditions stated was also destroyed in part by pepsin, another proteolytic enzyme, but it is evident that this enzyme acts somewhat differently to trypsin.

As regards the ability of different strains of tubercle bacilli to produce active tuberculins on different synthetic media: Within certain limits, the weight of growth in conjunction with age may be taken as a rough indication of tuberculin activity and pathogenic strains appear to produce more potent tuberculin generally than do greatly attenuated or avirulent strains, but irrespective of virulence, certain strains appear to produce more potent tuberculins, weight for weight of bacillary growth, than do others. The suitability of synthetic media employed for the production of tuberculin is, of course, an indirect factor governing potency for as was shown in study V the kind of medium supporting the more profuse growths produced the more potent tuberculins. The technique employed in the preparation of tuberculin also appeared to have an important bearing on potency, particularly so as regards indicated activity by intracutaneous and complement fixation methods as was revealed in study VI.

The stability of the active properties of crude tuberculin is generally accepted and as a natural sequence it is, I believe, generally assumed that a sample once standardized would retain its active principles unchanged for an indefinite period (8) if kept in the crude state, and could therefore be employed as a standard by which other samples might be compared and standardized. The indications in the foregoing studies, however, are that this assumption is not true in every instance in so far as the intracutaneous activity and complement fixation reactions of concentrated synthetic tuberculins are concerned, as in most samples studied the molecule responsible for these two reactions underwent changes when kept at room temperature for any considerable period, causing a reduction in their activities by both test methods. It is true, however, that lethal activity may be retained indefinitely and may be even enhanced under these conditions, but as shown the lethal activity *per se* may not be always an accurate gauge of the skin reacting properties of tuberculin.

This change of intracutaneous activity in concentrated synthetic tuberculin is apparently slow and owing to the large excess usually employed when determining activity in tuberculous animals by this method, may pass unnoticed, or if noted, be attributed to the lack of sensitiveness on the part of the test animals. This latter suggestion provokes further consideration and focuses discussion on the degree of sensitiveness necessary to obtain adequate reactions in test animals when determining potency by the intracutaneous test, in comparison to sensitiveness required for lethal potency determinations.

The lethal dose method as devised by Schroder & Brett (6) requires that the test be performed within a short period after sensitiveness to tuberculin has been established, as determined by death in a certain percentage of test animals following the intra-abdominal inoculation with a predetermined dose of tuberculin. In advocating the intracutaneous test for determining potency Aronson (7) indicated a definite procedure for obtaining suitably sensitized animals, but it is doubtful whether the importance of this requisite has been fully realized by all workers employing this method for determining potency.

Sensitiveness to the lethal method although irregular in individual test animals is generally progressive, that is to say, the longer the period after infection the more sensitive test animals become, thereby requiring a smaller dose of tuberculin to cause death. This may be illustrated simply by a summary of unreported but repeated personal observations which have shown that if guinea pigs from a group of animals infected about six weeks previously required a minimum dose of 0·25 c.c. of a potent concentrated tuberculin per 500 grammes live weight, inoculated intra-abdominally, to cause death in four of six test animals, guinea pigs of the same group, at from four to six months after infection and which were not previously tuberculinized, would often succumb when inoculated intracutaneously with one tenth of the dose of the same tuberculin which was originally required to cause the percentage of fatalities mentioned by intra-abdominal inoculation.

Sensitiveness to the intracutaneous method may be, like that found by the lethal method, irregular in individuals of a group of tuberculous guinea pigs infected at the same time, as was indicated in study III. On the other hand, it is only progressive within certain limits of time, which apparently varies in relation to the kind and amount of the infective dose and to the individual idiosyncrasies of different animals. The general indications from my observations are that tuberculin sensitiveness in tuberculous guinea pigs after it reaches its zenith recedes rather irregularly, which is in accord with the published results of Parish & Okell (8). Unlike these authors, however, I was unable to determine as they did the most suitable time after infection, which they consider to be three weeks, for determining tuberculin potency by the intracutaneous method, since as previously stated this is a variable dependent on several factors. With this exception the literature reviewed did not indicate the exact time after infection when ideal sensitiveness for determining tuberculin potency by the intracutaneous method may be expected in tuberculous guinea pigs.

It is therefore not improbable that a particular tuberculin meeting the potency requirements, as for instance, by Aronson's technique, in one group of infected animals, would be found inadequate in another group infected with another strain, tested at the same time after infection as the first group or in the first group of infected animals tested at a different time or even in sub-groups of one infected group tested at one time. In the final analysis therefore determination of potency by intracutaneous titration is only relative and should therefore be performed in comparison with a "Standard," which, however, as has been indicated is liable to change.

A word with respect to the grading and recording of tuberculin reactions obtained by intracutaneous test method in tuberculous guinea pigs. It is apparent from the literature that observers have considered necrosis, when present in conjunction with hyperemia swelling and induration to be an indication of a higher grade reaction than when absent and consequently the sample of tuberculin causing it more potent than another that did not evince it. This predication appears to be not without pitfalls, for as was shown in study XII, technique is a factor of no little importance governing skin necrosis following intracutaneous inoculation of tuberculin. Other observers have recorded reactions in terms of precise measurement *per se* or in comparison with normal skin thickness, which denotes an accuracy that is often lacking. In my hands either of these methods

have shown appreciable differences in reactions at different sites in the same test animal, with similar test doses of a single tuberculin, which, as in case of skin necrosis, was undoubtedly due to technique and they were in no way more reliable than estimating the reactions in terms of symbols.

The observation of Calmette (9) that purified tuberculins will not serve as an antigen by complement fixation methods and employed in support of criticism of this test as a method for determining tuberculin potency, appears to be inaccurate for, as indicated in study IV, tuberculins prepared in synthetic media and then dialysed were found to have antigenic properties suitable for titration and the explanation of Calmette's observations may be found in the particular technique he employed or to the possibility that the tuberculin he studied and reported on in this respect had been hydrolysed to such an extent that it contained only the lethal principle. Other criticisms of the complement fixation method have been dealt with also and have been more or less satisfied.

The technique as employed for the determination of tuberculin potency by complement fixation methods cannot be considered difficult, although accuracy and an understanding of the fundamentals underlying the method are essential and indeed, the only difficulty that may be encountered is in connection with procuring suitable anti-sera, for, as indicated in the study covering the production of this reagent, any anti-serum from a tuberculous animal because it will deviate complement in the presence of a tuberculo antigen, does not guarantee that it will be adequate for titrating tuberculin. Although no infallible method has been as yet devised whereby this all important reagent may be always obtained, if the general principles relative to its production as given in study X are adhered to, a suitable reagent may be obtained from most horses and mules.

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