

THE EFFECT OF META-iodo BENZYL CINNAMATE
ON THE COURSE OF EXPERIMENTAL
TUBERCULOSIS IN THE GUINEA PIG.

by

Howard J. Horn, B.A.

A thesis submitted for the
Degree of Master of Arts in the
Department of Bacteriology

September 30, 1935.

I.--Introduction.

In a discussion on various attempts at chemotherapy in Tuberculosis Calmette makes the following statements; "It must be recognized that up to the present, despite the great number of attempts made to discover among chemical agents a substance capable of arresting the development of experimental tuberculosis in the guinea pig and the rabbit, these efforts have been in vain. But this is not a reason for discouragement. Certain attempts among those which we have cited deserve further attention. It will be desirable, for example, to give further consideration to those with iodized compounds which, if they do not appear full of promise, give, nevertheless, some definitely favorable results.

"We must always remember that it is useless, perhaps it may be dangerous, to inject at random, as has been too often done, such and such a chemical into patients with the vague hope of discovering a specific activity. This is a practise which should be condemned. Experimentation alone, methodically conducted upon animals sensitive to tuberculosis, will enable us to explore with profit the immense perspectives that chemotherapy offers."

The Department of Chemistry at this University has for several years, been building up different combinations

2.

of certain of the compounds which seem to have given promise of chemotherapeutic value in tuberculosis. These compounds include urea, benzyl alcohol, cinnamyl chloride, iodine, and allied compounds. The papers of the Department of Chemistry discuss in full the compounds synthesized and the methods of synthesis employed.

The Department of Bacteriology was then approached with a view to determining the therapeutic value of the drugs synthesized. This paper being, it is hoped, the first of a series on the behavior in vivo of these compounds, is largely devoted to a description of the technique and methods employed, although a complete description of the behavior of one drug is included.

Bearing in mind the tenets of Calmette it was determined to cover the ground slowly and thoroughly. It was decided to use large populations of guinea pigs for the experiments, rather than inoculating a few at random, and also to use a number of controls equal to that of the test population.

There was first to be discovered the best method by which to administer the drugs.

Method of Administration of Chemicals:

The chemicals to be used in a preliminary series were benzyl cinnamate, meta-iodo benzyl cinnamate, and ethyl meta-iodo cinnamate. These compounds (as can be found by reference to the papers of the Chemistry Department on their preparations) are all solids at room temperature (23°C) and insoluble in water. Their solubility in substances usually employed as solvents for injection is very low. Emulsification without the use of an emulsifying agent was not possible: thus it was thought best not to use the drug in emulsified form as the protective colloid used as the emulsifying agent might very easily interfere with the action of the drug itself.

It was found, however, that although the drugs did not melt until considerably above blood heat (37°C) they could be cooled while molten to blood heat without solidifying. It was therefore decided to inoculate the pure drug in molten state at 38°C . thus making sure that the effects, if any, would be due to the chemical and to that alone.

Intracardiac inoculation was first tried as being the most direct method. Two difficulties were encountered, however, which rendered this system impractical. The first difficulty was that although recovery of uninoculated guinea

pigs from the anaesthetic used, whether ether or chloroform, was normal, the recovery of guinea pigs inoculated with the drug while under anaesthetic was very slow, and often unsuccessful. Since these drugs are highly soluble in both ether and chloroform this phenomenon may have been due to some sort of loose chemical, or even physical, combination between the drug and the anaesthetic. Certain individual guinea pigs, however, did recover from inoculations of as much as 0.75 c.c. benzyl cinnamate or meta-iodo benzyl cinnamate or 0.5 c.c. ethyl meta-iodo cinnamate. The other difficulty encountered, however, was such as to make this system of inoculation unsatisfactory. The needle of gauge large enough to pass the molten chemicals was not small enough in outside diameter to ensure that the heart muscle would close the puncture, and the deaths by hemorrhage were thus far too frequent to make practicable inoculation by this method. The necessity for a needle of large gauge also made intravenous inoculation impossible.

Intraperitoneal inoculation of the various compounds was then attempted and found to be highly satisfactory. The guinea pigs stood inoculations of up to 1.0 c.c. each of the chemicals without any loss of weight or other symptoms of distress.

The technique was as follows: the chemical to be inoculated was placed in a sterile container and heated at 60°C. until liquid. The molten drug was then drawn up into a sterile syringe with a needle of 22 gauge and inoculated intraperitoneally into the guinea pig. The guinea pig had previously been prepared by clipping a space about one inch square on its abdomen and wiping with both alcohol and iodine solution.

In finally determining the route by which the drug was to be administered, consideration was also given to the fact that the drugs are highly insoluble. If the intracardiac or intravenous route were chosen, the total amount of comparatively insoluble material which could safely be given was obviously very small and the resulting assimilation of the soluble portion, if any, into the system would be negligible. On the other hand, if injections were given intraperitoneally large quantities of the drug could be administered, with the result that a greater total amount might possibly be assimilated into the system.

Care of Experimental Guinea Pigs.

The experimental guinea pigs were kept in individual cages and weighed, fed, and cleaned at regular intervals. In this group of experiments it was decided for convenience' sake to perform these operations on Mondays, 9A.M. Wed-

nesdays 1:30 P.M., and Fridays 5:30 P.M.

The ration, given after weighing consisted of 300gms. green feed (moist)--lettuce, cabbage, and cauliflower leaves--and 85 gms. crushed oats. No water was given as the fresh greens were found to be sufficient as a source of moisture. The cages in every case were cleaned and washed thoroughly after each weighing but before feeding.

It was found that by feeding at longer intervals than one day, the weights did not show as great a day-to-day variation, since there was time for evacuation of the intestines after feeding.

Preparation of Myc. tuberculosis for Inoculation.

The tubercle bacilli used were from a virulent culture, freshly isolated from sputum at the Vancouver General Hospital through the kindness of Dr. H.H. Pitts, Director of the Laboratories. The cultures were all grown on Petragnani's medium at 37 C.

A 15--day culture was washed off the medium with sterile saline (0.87%), using a sterile Pasteur pipette. The suspension was well mixed by continued drawing up and expelling with the pipette, and then allowing the large lumps to settle out before removal from the tube. The resulting suspension was then transferred to another sterile tube and diluted with sterile saline to a turbidity equivalent to MacFarland Standard #4. After much experimentation it was

found that 0.25 c.c. of a 1/20 dilution of this turbidity inoculated intraperitoneally would cause the death of a guinea pig after approximately 6 weeks. This, then, was the infecting dose used for the preliminary tests.

For the more conclusive experiment the culture used was from the Tranquille Sanatorium, isolated from pleural fluid, third subculture. This was a more slowly growing organism and necessitated the use of an 86 day culture for maximum growth.

After further experimentation it was found best to allow the suspension of turbidity #4 to settle for exactly 10 minutes and then make the 1/20 dilution from the supernatant suspension. This removed the majority of clumps of bacilli, and gave a much finer suspension, thus increasing the period before death to approximately 12 weeks. This therefore gave any possible therapeutic powers of the drug a much better opportunity.

Inoculation, whether of drug or tubercle bacilli, was for convenience' sake always given before feeding, a period at which the intestines would be comparatively empty.

Preliminary Experiments.

To determine if the drugs had any prophylactic properties it was decided to inoculate small numbers of guinea pigs with the drug and tubercle bacilli simultaneously. It

8.

was thought that this perhaps would give some lead as to the relative value of the drugs and help to decide which of several should be used in the more extensive experiment. The following table shows the details of the preliminary experiments

Number of guinea pigs	Compound inoculated	Quantity of compound	Quantity of suspension of tubercle bacilli inoculated
4	Benzyl cinnamate	1.0c.c.	0.25 c.c.
4	Meta-iodo benzyl cinnamate	1.0 c.c.	0.25 c.c.
4	Ethyl meta-iodo cinnamate	0.5 c.c.	0.25 c.c.
4	None--Controls		0.25 c.c.

Of the controls, three averaged 47 days before death: the remaining control, however, remained alive for 77 days. The guinea pigs inoculated with ethyl meta-iodo cinnamate also lived for an average of 47 days after inoculation. The animals inoculated with benzyl cinnamate averaged 62

days, one remaining alive for 73 days. The guinea pigs inoculated with metapiodo benzyl cinnamate gave by far the most promising results, averaging 62 days, one however, remaining alive for 93 days.

Due to the one control which was living at 77 days after inoculation these results did not seem to be even indicative of any conclusions. This guinea pig, however, on autopsy, showed an ear-puncture, proving that at some time this animal had been ear-tagged. After a check-up of animals it was found that this was a guinea pig which had been tagged when inoculated intracardially 71 days previous to the inoculation with tubercle bacilli with 0.7 c.c. benzyl cinnamate for a toxicity test. This, then, caused the elimination of this animal: thus the experiments gave some indication of slightly better prophylaxis from meta-iodo benzyl cinnamate than from benzyl cinnamate.

Such inconclusive results definitely upheld the necessity of employing much larger populations for the next experiment. For the large scale experiment it was decided to try meta-iodo benzyl cinnamate as it had given some indication of slightly better prophylaxis than benzyl cinnamate. It was also thought that it would perhaps combine the supposed therapeutic properties of both benzyl cinnamate and iodine.

The first method used by the Chemistry Department for

the synthesis of this drug was successful for small quantities only. As it was calculated that at least 100 gms. of the compound would be required, the writer assisted the investigator in the Department of Chemistry for several months in devising a new method for quantity production.

Final Experiment with Meta-iodo Benzyl
Cinnamate.

Method of Recording Guinea Pig Weights.

Due to the highly inconclusive results with smaller populations in the preliminary work it was decided to use a population of 20 guinea pigs for the final experiment, with an additional 20 guinea pigs for the control. Each population consisted of 8 males and 12 females, the control animals averaging 424 gms. and the test animals 419 gms. It may be well to say here that the proportion of males to females was not due to any statistical requirement, but to the fact that the young animals on hand happened to be so divided. The animals were to be fed and weighed, following the normal routine for a considerable period without any inoculations. The average weight of each population at each weighing was to be carried into a moving average. This was done in the following manner--for each population the total weight is divided by the number of guinea pigs, giving the weight of the average guinea pig for that day. These weights were then

added in overlapping groups of three and the mean taken, thus giving a moving average. This method tends to smooth out the curve, since it eliminates to a large extent the daily fluctuations. The moving averages of the two populations were plotted against the time on semilogarithmic paper. The complete experiment lasted from Oct. 22, 1934 to Aug. 12, 1935,

Inoculations.

With Meta-iodo Benzyl Cinnamate.

In order to give the drug every possible chance it was decided to inoculate the drug in 0.5 c.c. quantities at several different periods. The guinea pigs were thus inoculated with the drug 14 days, 7 days, and 3 days before receiving the inoculation of the tubercle bacilli and 2, 9, and 16 days after.

It is interesting to note on the curve that the drug had seemingly no effect up to the time of the inoculation with Myc. tuberculosis. On receiving the tubercle bacilli, the animals immediately began to drop in weight, recovering slightly and then dropping again at each further inoculation with the drug. It was for this reason that the drug was discontinued 16 days after the tubercle bacilli inoculation, as the guinea pigs were becoming rough-haired and emaciated and showing further signs of distress.

With Tubercle Bacilli.

As described before the inoculation used for both

populations was 0.25 c.c. of a 1/20 dilution of a turbidity equal to Mac Farland #4, using the supernatant liquid for dilution after 10 minutes settling.

Results.

Statistically the results were entirely negative. Eleven of the treated animals died of tuberculosis before 111 days, whereas only eight of the controls died over the same period. The remaining guinea pigs were then killed and autopsied, all showing definite symptoms of tuberculosis.

The knowledge that these deaths were specifically due to tuberculosis infection, is owing to the kind co-operation of Dr. H.M. Pitts, Pathologist and Director of Laboratories at the Vancouver General Hospital. Dr. Pitts performed autopsy and histological examination on all guinea pigs used in the experiments.

It would seem therefore, from this, that the drug is of no value in tuberculosis prophylaxis. In fact, from a study of the weight curves it would seem that the drug has a detrimental effect in the presence of tuberculosis, as explained in the previous section.

In every case, it was found that the treated guinea pigs on autopsy showed extensive intraperitoneal adhesions, whereas the undrugged guinea pigs did not. The adhesions therefore were doubtless due to the irritating action of the drug.

Discussion.

The one valid conclusion which may be drawn from this

experiment is that meta-iodo benzyl cinnamate is of no therapeutic value in experimental tuberculosis. Large populations were used; a minimal inoculum of tubercle bacilli was given; drug inoculations were so spaced as to give ample opportunity for both prophylactic and therapeutic action.

It is interesting, however, to speculate upon the mechanism of the reaction of the drugged pigs following inoculation of the tubercle bacilli.

It is obvious that this cannot be the reaction of the drug on tuberculous lesions as such. It might be explained by accumulative traumatic effect of continued inoculations upon the peritoneum of the guinea pig.

On the other hand it is possible that the drug had sensitized the animals to the tuberculous toxins or even to the actual protein of the organism. "Sensitized" is used here in the sense of a chemical sensitization rather than with any reference to allergy.

References.

Since this paper, in the main, concerns the action of a chemical compound which has not been hitherto prepared, references alluding directly to the subject matter are naturally unobtainable. For references to work of a similar nature, the reader is referred to the following sources:--

Index-Catalogue of the Library of the Surgeon General's

Office, United States Army: Third Series, Vol.X, 1932,--See

Tuberculosis (Experimental, Treatment of) p.527.

Tuberculosis (Pulmonary, Treatment of)p.581--583.

Tuberculosis (Pulmonary, Treatment of)with calcium
p.592.

Tuberculosis (Pulmonary, Treatment of) by chemotherapy
p.592--593.

Tuberculosis (Pulmonary, Treatment of) by gold solutions
p.593

Tuberculosis (Pulmonary, Treatment of) by inhalation
therapy. p.596

Tuberculosis (Pulmonary, Treatment of) with iodine.
p.596

Tuberculosis (Pulmonary, Treatment of) with sanocrysin.
p.599.

Tuberculosis (Pulmonary, Treatment of) with silicic
acid. p.600.

Tuberculosis (Pulmonary, Treatment of) with sugar
p.600.

Authorized English Edition--"Tubercle Bacillus Infection and

Tuberculosis in Man and Animals" by Albert Calmette--

Williams and Wilkins Co., Baltimore, 1923.--with special

reference to Chapter XLIII. "Attempts at Chemotherapy in
Tuberculosis"

Zur Jodbehandlung der Lungentuberkulose; A. Sylla; Beitr.

z. Klin. d. Tuberk. May, 1932, lxxx,51.

The use of Iodine and certain Iodine compounds in exper-

imental tuberculosis. G. Knaysi, J. Inf. Dis. March, 1932, 1, 261.

Treatment of pulmonary tuberculosis on lines of mineral deficiency. K. Fraser, Brit. M. J. May 24, 1930, #3620, 946.

Appendix.

Since there appear to be several modifications of Petraghani's medium for the cultivation of Myc. tuberculosis, it was deemed advisable to give here the formula followed in this particular experiment. The strains of Myc. tuberculosis used would give satisfactory growth on this medium in less than one-half the time required on any of the more common media for tubercle bacillus cultivation.

Milk.....	900 c.c.
Potato flour.....	36 grams
Peptone.....	6 grams
Potato--egg-sized pieces.....	6
Eggs--whole.....	24
Egg yolks.....	6
Glycerine.....	70 c.c.
Malachite green--2% aqueous solution.....	60 c.c.

Grind potatoes with fine meat grinder. Mix all ingredients. This mixture is kept in a boiling water bath

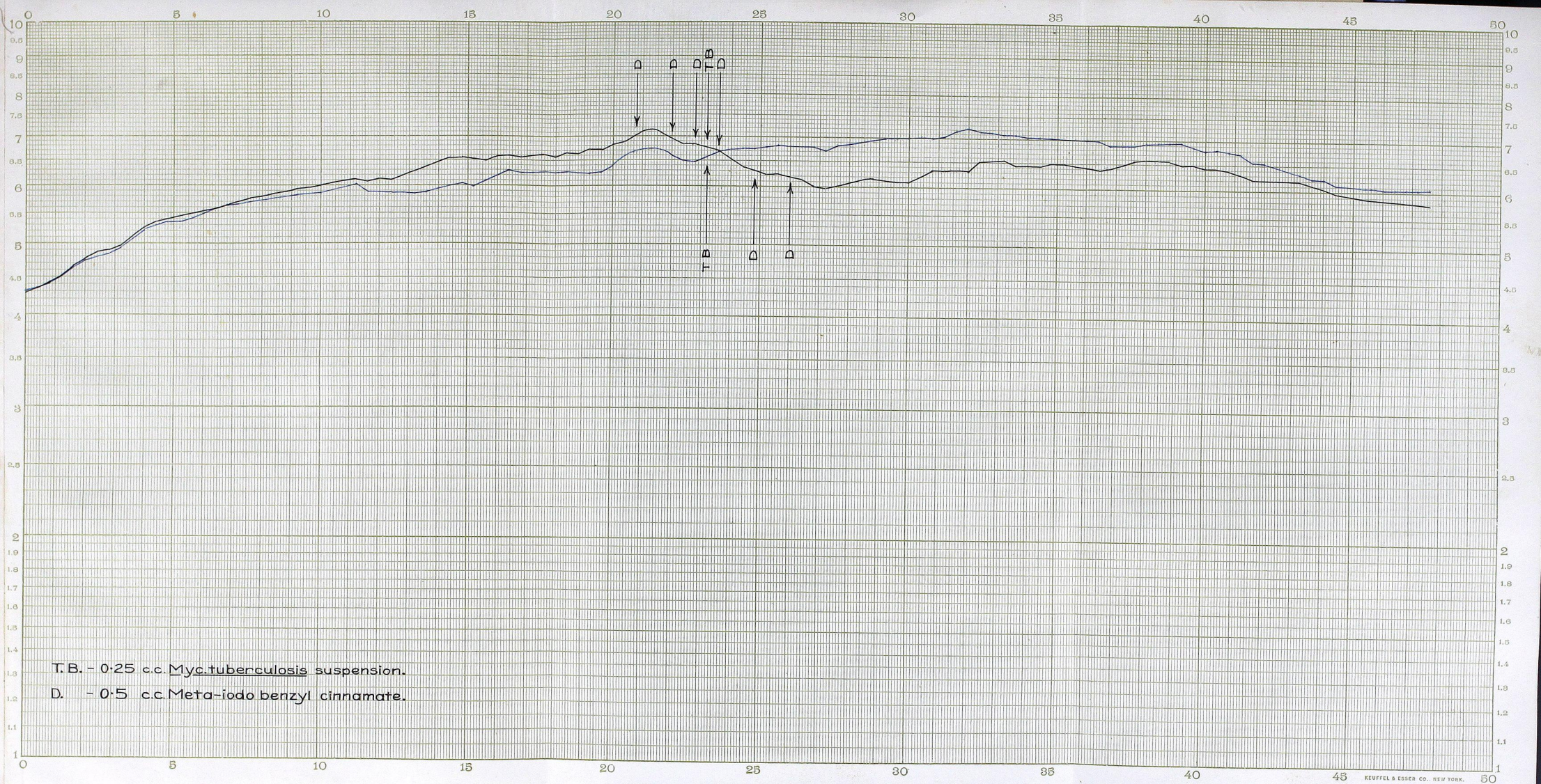
16.

with frequent stirring until it becomes sticky. After this it is left in the water bath for 1 to 2 hours. After cooling to 50 C. add 24 whole eggs and 6 egg yolks and 70 c.c. glycerine. Then add 60 c.c. 2% aqueous solution malachite green. The whole mixture is filtered through sterile gauze, tubed for slants and placed in an Arnold in a slanting position.

Incubate--first day 1/2 hour at 70 to 75 C.

next day 1/2 hour at 80 C.

last day 1/2 hour at 80 C.



T.B. - 0.25 c.c. *Myc. tuberculosis* suspension.

D. - 0.5 c.c. Meta-iodo benzyl cinnamate.