FERMENT-INHIBITING SUBSTANCES IN TUBERCLE BACILLI.

STUDIES ON FERMENT ACTION. XI.*

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A great deal of work has been done in the attempt to explain the processes that lead to the production of caseation in tuberculosis. By some it has been ascribed to anemia, the tubercle being avascular, and by others to the action of toxins derived from the tubercle bacilli.

Auclair (1) believes that the caseation is due to specific toxins of the nature of fat, which are soluble in ether, chloroform, benzine, and xylol. He treated tubercle bacilli with one or more of the above agents, evaporated the solvents, and suspended the extracted substances in water. These emulsions, when injected into the subcutaneous tissues of animals, produced typical caseous abscesses. When injected into the trachea of guinea pigs, caseous areas appeared in the lungs.

Schmoll (2) analyzed caseous material and found it almost free from proteoses, which indicates that autolysis is slight; but he does not explain the lack of autolysis. The work of Schmoll has been confirmed by others, and we have obtained similar results.

Anemia due to the occlusion of the blood vessels may be an important factor in causing caseation in the chronic forms of tuberculosis where the areas are surrounded by a layer of connective tissue. However, in acute caseous pneumonia in which there is a catarrhal exudate rapidly becoming caseous, some other explanation appears to be necessary. In such an exudate there are numerous cells known to contain ferments, yet these die and disintegrate, and, as far as can be determined, the ferments remain inactive. We must therefore look for some other factor to explain the process of caseation, and this we believe we have found in the tubercle bacillus, a ferment-inhibiting substance. We observed two years ago that the

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antitryptic action of sera was lost or greatly decreased when the sera were preserved with chloroform. This observation suggested to us the possibility that the antiferment properties of the blood were due to substances of the nature of lipoids, and the lack of autolysis in caseation and in anemic infarcts to substances of a similar nature.

With this hypothesis we decided to study the influence of the ether-soluble substances of tubercle bacilli on proteolysis. It has been shown by numerous investigators that the ether-soluble substances constitute 25 to 35 per cent. by weight of the bacilli, and of these extracts about 17 per cent. are composed of fatty acids. Both human and bovine tubercle bacilli were used in the work.[1]

Our first experiments were conducted with ether- and alcohol-soluble substances obtained from the tubercle bacilli. After the solvent had been evaporated, the material remaining was taken up in methyl or ethyl alcohol and suspended in a 0.9 per cent. solution of sodium chloride. In some experiments the emulsion was used just as prepared; in others the alcohol was first evaporated at low temperatures. The emulsion, in various dilutions, was then mixed with trypsin, incubated for thirty minutes, and the casein added. As far as we could determine there was no inhibition of ferment action.

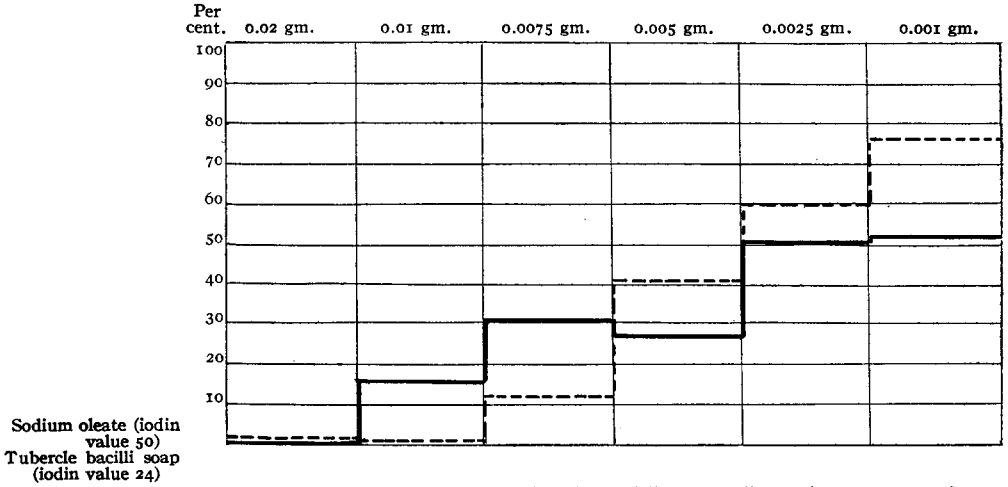
In the preliminary experiments we used the Fuld-Gross technique, but, for reasons given in our preceding paper (3), we soon discarded it in favor of the following. After incubating the mixtures for a certain period of time, they were acidified with a solution containing 10 per cent. acetic acid and 20 per cent. sodium chloride. The tubes were then placed in boiling water for five to ten minutes, filtered through kaolin to remove the coagulated protein, and the incoagulable nitrogen was determined according to the method recommended by Folin (4).

This experiment failed to demonstrate the presence of an inhibiting agent. This may have been due to the agent being in an emulsion and not a solution, thus preventing its coming into intimate con-

[1] We wish to thank Dr. Hitchens, of Glenolden, Pa., and Dr. Grund, of the New York Board of Health, for supplying us with large amounts of human and bovine tubercle bacilli.

tact with the ferment. We then fractionated the extracted material so that at least a part, the fatty acids, might be prepared in such a manner that they could be brought into solution. The material was dissolved in ether and precipitated with acetone. The acetone fraction was evaporated to dryness on the water bath and the resulting substance saponified with alcoholic potash. The soap was dissolved in water and repeatedly extracted with petroleum ether in order to remove any unsaponifiable matter. The acids were then liberated with hydrochloric acid, taken up with ether, washed with water, and resaponified.

The ferments were prepared and standardized according to the

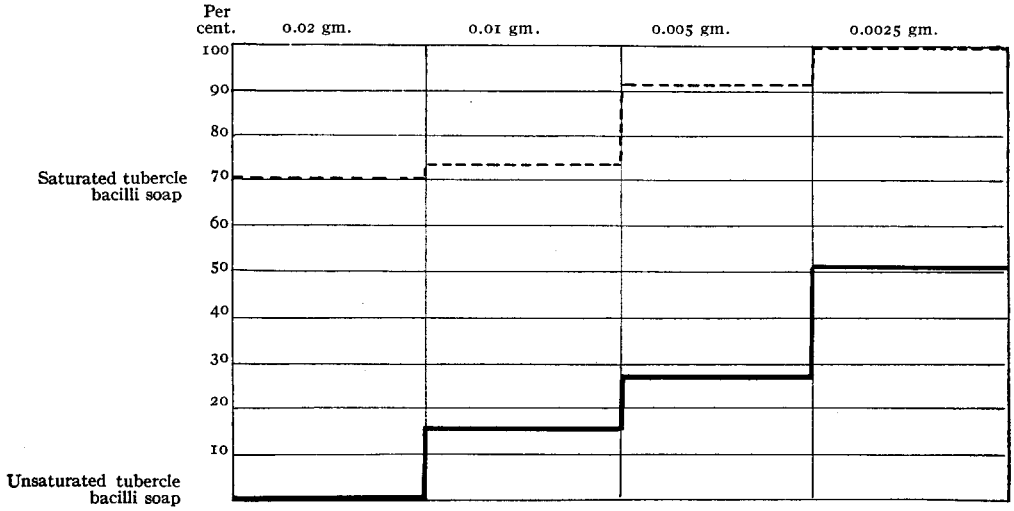


TEXT-FIG. 1. Effect of soaps of tubercle bacilli and sodium oleate on tryptic digestion.

method described in our previous paper (5). The soaps, in 1 per cent. solutions, were mixed with trypsin in varying amounts, incubated for thirty minutes, and the casein was then added. After the final incubation of two hours, the coagulable protein was removed and the incoagulable nitrogen determined. Text-figure 1 shows the results obtained with the soap prepared from extractives of the tubercle bacilli. A soap prepared from olive oil was used as a control. The dotted and heavy black lines indicate the percentage of digestion present in the tubes containing soaps as compared with the controls containing trypsin and casein alone.

The results of this experiment show that in the final dilutions the soap prepared from tubercle bacilli is more active as an inhibiting agent than the one prepared from olive oil. The iodin value of the soap prepared from the tubercle bacilli was 24, of the olive oil soap 50. The relation that the iodin value of the soaps bears to their activity as inhibiting agents was discussed in our previous report. It will be dealt with here in relation to the one prepared from tubercle bacilli.

In the previous paper (5) on the influence of soaps of the fatty acids on inhibition of ferment action, we demonstrated that the unsaturated fatty acids were the active agents, while the saturated fatty acids exerted but little if any inhibiting action. We were unable to obtain a complete separation of the saturated from the unsaturated acids and so could not remove entirely the inhibiting



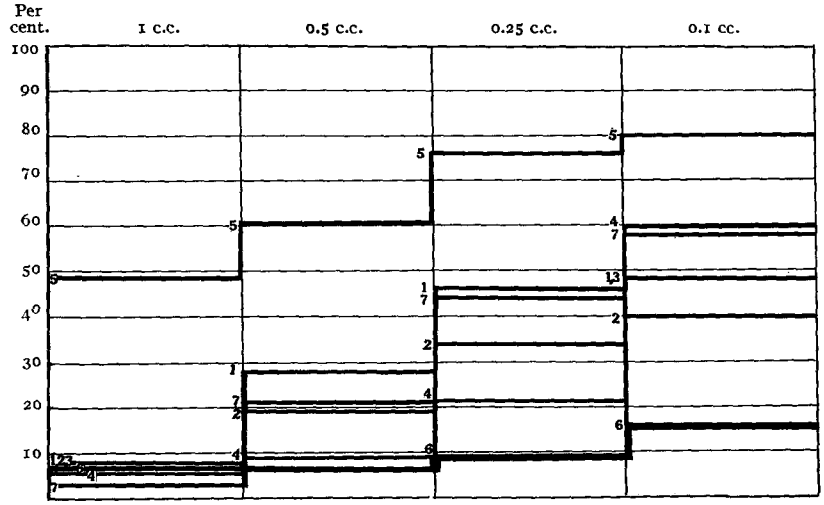
TEXT-FIG. 2. Effect of saturated and unsaturated soaps of tubercle bacilli on tryptic digestion.

action, but the differences in their activity following their partial separation by the ether-lead soap method were so striking as to be conclusive. The soaps of the saturated fatty acids tested, sodium stearate and sodium palmitate, were inactive.

We undertook to determine if the same is true of the soaps pre-

pared from tubercle bacilli. Lead soaps were prepared according to the usual method. The acids were set free with hydrochloric acid, washed, resaponified with sodium alcoholate, and evaporated to dryness. Text-figure 2 gives the results obtained with soaps prepared from saturated and unsaturated fatty acids.

The results obtained with the ether-soluble and insoluble fractions are as conclusive as those described in the preceding report for other soaps. The ether-lead soap method does not give a complete separation of the saturated and unsaturated fatty acids, but the separation is sufficient to demonstrate which is the active fraction.



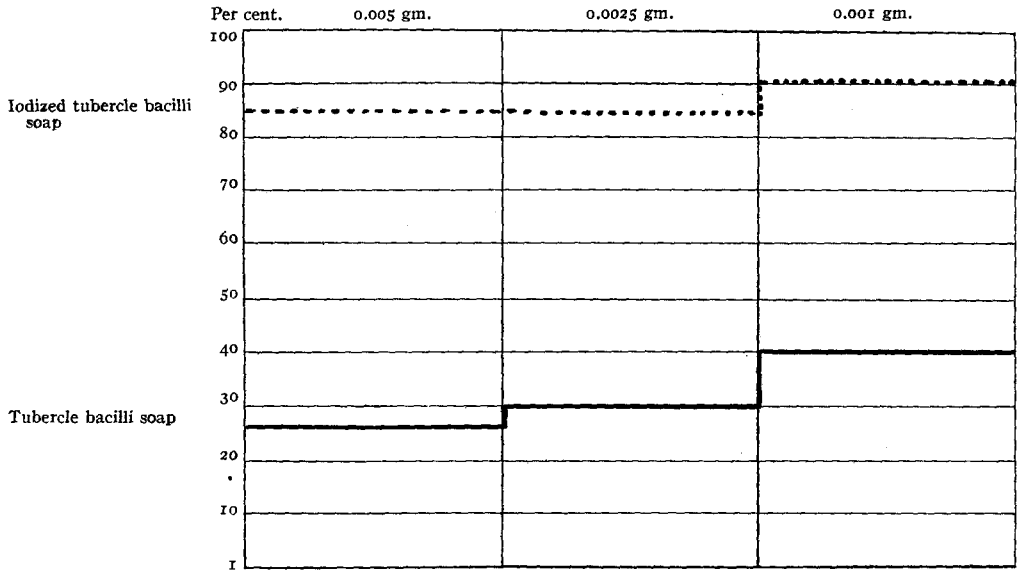
1 = hempseed soap.	Iodin value 100.	Solution 1.0 per cent.
2 = linseed soap.	Iodin value 100.	Solution 1.0 per cent.
3 = croton soap.	Iodin value 61.	Solution 1.6 per cent.
4 = olive oil soap.	Iodin value 44.	Solution 2.2 per cent.
5 = castor oil soap.	Iodin value 35.	Solution 3.0 per cent.
6 = tubercle bacilli soap.	Iodin value 25.	Solution 4.0 per cent.
7 = cod-liver oil soap.	Iodin value 92.	Solution 1.1 per cent.

TEXT-FIG. 3. Effect of soaps of tubercle bacilli and various other soaps on tryptic digestion.

We reported previously that, with one exception, the activity of the soaps of the unsaturated fatty acids as inhibiting agents was proportionate to their iodin value. We did not include in the experiment described the soap prepared from the extractives of

the tubercle bacilli. This is shown in text-figure 3 in comparison with the other soaps which we have been using. After the iodin value of the respective soaps had been determined, they were made up in such strengths that one cubic centimeter of each had approximately the same iodin value.

A study of text-figure 3 shows that of all the soaps investigated the one prepared from tubercle bacilli is the most active in preventing enzyme action. We had already observed that it was as active as the other soaps in 1 per cent. solutions and were not surprised to find it more so when made up according to its iodin value.



TEXT-FIG. 4. Effect of saturation of unsaturated soaps of tubercle bacilli with iodin.

The method used by us in extracting the acids from the bacilli and in preparing the soap does not give any indication of the quantity originally present. On the contrary, it offered every opportunity for the unsaturated acids to become saturated, thus lowering its iodin value and reducing its activity as an inhibiting agent.

In the previous report we showed that the activity of the soaps of the unsaturated fatty acids could be destroyed by saturation with iodin. The soaps so treated did not become entirely inactive,

but this may have been due either to the presence of some free iodin or to incomplete saturation. It is of importance to determine if the soaps prepared from tubercle bacilli are similarly affected when treated with iodin. To five cubic centimeters of a 1 per cent. solution of the soap was added 0.05 of a gram of iodin, and a few crystals of iodide of potassium, and the mixture was permitted to stand over night. The following morning it was shaken repeatedly with chloroform, until fresh portions of the latter remained clear, showing that there was no more free iodin. Text-figure 4 shows the effect of saturating the soap with iodin.

The soap used in this experiment was old and therefore not very active, but the effect of treatment with iodin was evident. The experiment was repeated several times and a similar result obtained in each instance.

Numerous chemicals have been studied in the attempt to find one that will be effective as a therapeutic agent in tuberculosis. Such an agent, in order to be of value, must combine with some constituent of the tubercle bacillus, and our experiments indicate that iodin as a component of the chemical substance may be the means of bringing about such a combination.

We do not know the nature of the unsaturated fatty acids exerting the inhibiting action, but their isolation and identification and further work on their inhibiting action are now under way.

Wells (6) obtained an average of 0.001 of a gram of fatty acids to one gram of caseous matter, which indicates the small amount of soap present in this substance. This finding has little bearing on the relation of the fatty acids of tubercle bacilli to caseation, as the influence of the soaps depends on the amount of ferment present and not on the quantity of protein. That small amounts of the soap are capable of inhibiting enzyme action is shown in text-figure 1, where it was found that in the tube containing 0.001 of a gram of soap the digestion was only half that obtained in the control containing no soap. The influence of soaps as inhibiting agents was well shown in our previous report in which it was demonstrated that the smallest quantity completely inhibiting the standard amount of trypsin materially influences the action of ten times that amount of ferment. In

addition, our experiments indicate that the ferments are destroyed as a result of combining with the soaps.

The soaps are probably formed from the neutral fats of the bacilli. The lipases hydrolyze the fats and the acids are then saponified. In partial support of this view we have the work of Klotz (7) who demonstrated lipases in tuberculous pus, and of Bergel (8) who states that the ferment is present in the lymphoid cells of tubercles, while Corper (9) found lipases in tubercle bacilli.

The demonstration that tubercle bacilli contain ferment-inhibiting substances brings us one step nearer a rational explanation of caseation in tuberculosis. The subject will be discussed more fully in a subsequent report.

CONCLUSIONS.

1. Tubercle bacilli contain unsaturated fatty acids which, when saponified, have the property of inhibiting the action of trypsin and leucoprotease.

2. In proportion to their iodin value these soaps are more active as inhibiting agents than the soaps prepared from linseed, olive, and cod-liver oils.

3. The activity of the soaps is dependent on the presence of unsaturated carbon bonds.

4. Saturation of the soaps with iodin destroys their inhibiting action.

5. Soaps probably play an important part in the production of the condition known as caseation in tuberculosis.

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