A STUDY OF THE FERMENTS AND FERMENT-INHIBITING SUBSTANCES IN TUBERCULOUS CASEOUS MATERIAL.

STUDIES ON FERMENT ACTION. XII.*

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In a previous paper (1) we reported that sodium soaps prepared from ether-soluble extractives of tubercle bacilli inhibit the action of trypsin and leucoprotease. The inhibiting action was found to depend on the presence of soaps of the unsaturated fatty acids, and could be destroyed by saturating the acids with iodin.

The results of this study appear to have a direct bearing on the development of caseation in tuberculosis. Caseation in tuberculosis is a form of coagulation necrosis in which the dead tissues rarely undergo autolysis, except as a result of secondary infection. Syphilis is the only infectious disease presenting a similar condition. In other instances of coagulation necrosis the dead tissues are soon removed by means of autolysis and phagocytosis. It appears, then, that substances having the property of preventing autolysis must be present in syphilitic and tuberculous tissues.

This view is confirmed by the work of Schmoll (2) who was unable to find any evidences of autolysis in caseous material. The results obtained by Auclair (3) can be explained in a similar manner. Auclair believes that caseation is due to specific toxins of the nature of fats which are soluble in ether, chloroform, and benzin. These extracts suspended in water produced typical caseous abscesses when injected into the subcutaneous tissues of animals, and caseous areas in the lungs when injected into the trachea of guinea pigs. No doubt the conditions observed by Auclair closely simulated caseous areas, but we doubt if they were due to the presence of specific toxins. It is more probable that his injections caused the usual inflammatory exudate which assumed the character of the caseous matter owing to the failure of autolysis.

The lack of autolysis in caseous material is similar to that observed in non-

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infected anemic infarcts. In the latter condition the dead tissue is finally removed by the phagocytic leucocytes which invade it from the surrounding tissues. The leucocytes also liberate ferments which aid in causing solution and absorption of the dead tissue. It is only in the large infarcts that softening occurs, and this is in the central part of the mass, while the periphery remains firm. Wiener (4) found that the autolytic enzymes act best in an acid medium, and Wells (5) believes that this explains the central softening in large infarcts. According to Wells, the acids at the periphery are neutralized by the blood plasma, so that autolysis is active only at the center.

Anemia, due to occlusion of the blood vessels, may be an important factor in causing caseation in the chronic forms of tuberculosis. However, in acute caseous pneumonia some other explanation seems necessary. Here we do not have the connective tissue reaction seen in the chronic forms of the disease, and we must therefore look for some other cause to explain the conditions present.

In a previous report (6) we showed that tubercle bacilli contain unsaturated fatty acids which, when saponified, are able to inhibit enzyme action. We believe that this observation explains the lack of autolysis in caseous matter, but it was considered necessary to make a careful study of caseous material in order to find out if ferments and ferment-inhibiting substances were present.

Schmoll's (7) failure to find evidences of autolysis in the caseous material from lymph glands indicates that ferment action has been inhibited from the start. This assumption is based not only upon Schmoll's work, but also upon our observation (8) that trypsin is destroyed when incubated for thirty minutes with soaps of the unsaturated fatty acids. If this view is correct, failure of proteolysis is due to destruction of the ferments by the soaps, but we must also consider the possibility of undestroyed ferments being present, though inactive, owing to the presence of inhibiting substances. It is unlikely that free ferments should be present in the caseous material obtained from chronic tuberculous lymph glands, but they may be present in a rapidly progressing caseous pneumonia with a marked inflammatory exudate. In the latter condition it is impossible to separate the caseous material from the inflammatory exudate, and so extracts of the caseous material will probably show the presence of some undestroyed ferments.

The caseous matter used in this study was obtained from tuberculous lymph glands and from several cases of rapidly progressing

caseous pneumonia.[1] The method used to determine the extent of digestion was that described in our previous reports. The mixture was made definitely acid with a solution composed of 10 per cent. acetic acid and 20 per cent. sodium chloride. The tubes were then placed in boiling water for five to ten minutes to coagulate the protein and the contents afterwards filtered through kaolin. The total incoagulable nitrogen was determined according to the method recommended by Folin.

CASEOUS LYMPH GLANDS.

The caseous matter derived from the lymph glands was spread in thin layers on glass plates and dried at temperatures below 45° C. After being dried the material was ground in a mortar to a fine powder. For the experimental work the emulsions were prepared in the proportions of one gram of caseous matter to 100 cubic centimeters of water. The material was examined to find out if ferments and ferment-inhibiting substances were present and also for evidences of autolysis. To determine the extent of autolysis a flask containing fifty cubic centimeters of the emulsion was placed in the incubator and left there for five days. Every day five cubic centimeters were removed and the amount of incoagulable nitrogen was determined. The incoagulable nitrogen obtained in five cubic centimeters of the emulsion on the fifth day was practically the same as that obtained on the first day. The results of this experiment indicate that either ferments are not present in the caseous matter, or their activity is suppressed. The freshly prepared emulsion was neutral in reaction, but became slightly acid after the second day. This is important as in the beginning the reaction was favorable to the ferments active in an alkaline or neutral reaction, and after the second day it was favorable to those active in an acid reaction, yet autolysis did not occur.

We obtained somewhat different results in a similar experiment conducted with caseous material from a lymph gland which had become secondarily infected. Autolysis occurred in a more marked

[1] We wish to thank Dr. F. Mathews of St. Mary's Free Hospital, Dr. Larkin of the New York Hospital, and Dr. Lamb of the Presbyterian Hospital for their kindness in furnishing us with material.

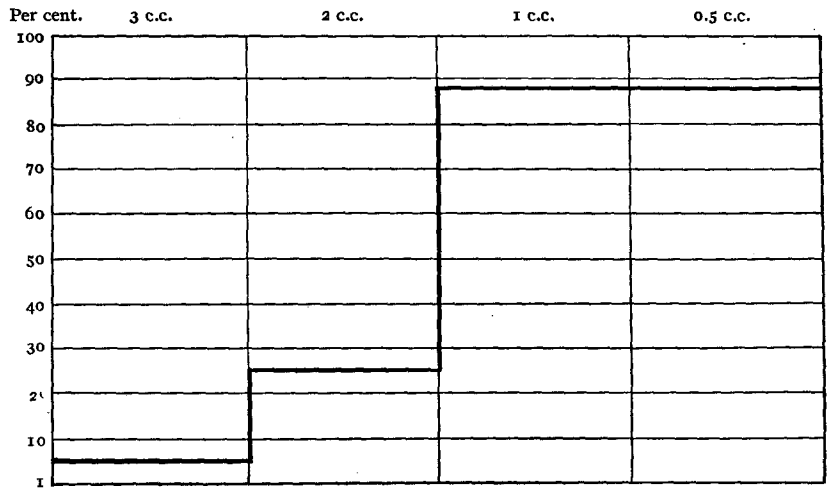
degree in an alkaline reaction, but was much more active in an acid reaction. In no instance have we been able to obtain evidences of autolysis in emulsions of uninfected caseous matter of lymph glands, either in acid or alkaline reactions. The lack of autolysis in the caseous material might have been due to the presence of soaps, and we therefore attempted to rule out this factor.

Two methods were used to determine the presence of undestroyed ferments. In the first the whole emulsion was treated with an excess of alcohol to which a small amount of hydrochloric acid had been added. Treated in this manner the ferments and protein were precipitated, and the fatty acids liberated by the hydrochloric acid were made soluble in the acid alcohol. The precipitate was then washed with alcohol and ether, and dried. The powder thus obtained was made into an emulsion with water, a portion made acid and another alkaline, and placed in the incubator for several days. The presence of ferments would have been shown by the degree of autolysis that occurred. In the second method the emulsion was first incubated for twenty-four hours in an alkaline solution in order to get the ferments and soap into solution. It was then centrifuged and the supernatant fluid treated as was the whole emulsion. In this instance the powder was made into an emulsion and tested against casein. In neither case were we able to demonstrate the presence of ferments.

We believe that the results of these experiments justify us in concluding that undestroyed ferments are rarely present in caseous lymph glands which have not become secondarily infected.

As practically all cells contain intracellular ferments, we must assume that substances were present which prevented autolysis. Our previous work indicated that these substances were probably soaps, therefore our next experiments were planned to see if this was the case. A fresh 1 per cent. emulsion was prepared and kept at room temperature for several hours. During this period it was shaken frequently. It was then centrifuged until it became clear. The supernatant fluid was now removed, made strongly alkaline with sodium hydrate, and the flask containing it placed in boiling water for several minutes. The fatty acids were then liberated with hydrochloric acid, taken up in ether, and resaponified.

The last step was repeated in order to free the soaps from unsaponifiable matter. The soaps were then tested for their enzyme-inhibiting action. They were mixed in various dilutions with trypsin and placed in the incubator at 37° C. for thirty minutes. The casein was then added and the mixture placed in the incubator again for about two hours. After the final incubation the total incoagulable nitrogen was determined according to Folin's method. Text-



TEXT-FIG. 1. Effect of the saponified extract from caseous lymph glands on tryptic digestion.

figure 1 shows the influence of soaps obtained from the caseous matter of lymph glands on the activity of trypsin.

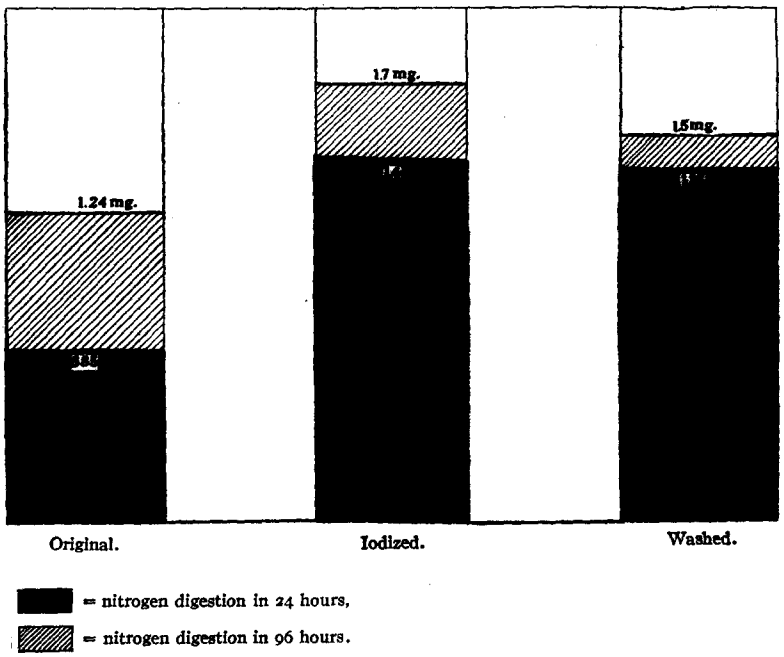
The soaps had been dissolved in sufficient water so that one cubic centimeter of the solution represented the amount of soap contained in one cubic centimeter of the original 1 per cent. emulsion. Three cubic centimeters of the emulsion, representing the soap contained in 0.03 of a gram of caseous matter, was sufficient to inhibit the action of the ferment. In the tube containing two cubic centimeters the digestion was only 25 per cent. of that obtained in the control tube containing no soaps.

The results obtained with the caseous material taken from a lymph gland which had become secondarily infected differ from those just described. Similar experiments were conducted with

this material, which was semifluid in consistence, and ferments active in alkaline and acid reactions were demonstrated. While autolysis occurred in both acid and alkaline reactions, it was more marked in the acid reaction, indicating that soaps were still present.

With this possibility in mind we decided to study the influence of iodin on the whole emulsion to see if it would accelerate autolysis. We have already shown (9) that treating the soaps of the unsaturated fatty acids with iodin destroys their ferment-inhibiting action, and it is therefore of interest to determine if similar results can be obtained with the native mixtures of protein and soaps.

The emulsion was divided into two portions, one of which was used as a control. To the other was added a mixture of iodin and iodide of potassium. The mixture was allowed to stand for several hours, and the excess of iodin was then removed by repeated extractions with chloroform. A third flask containing the washed precipitate was also used. To each of these flasks trypsin was



TEXT-FIG. 2. Effect of iodin on ferment-inhibiting substances present in caseous matter of lymph glands.

added. Text-figure 2 shows the amount of incoagulable nitrogen in two cubic centimeters of the emulsion after twenty-four and ninety-six hours' incubation at 37° C. The first column shows the amount of incoagulable nitrogen obtained after digesting the untreated emulsion with trypsin. The second column represents the amount of nitrogen obtained by digesting the iodized fraction, and the third the action of trypsin on the precipitate which had been freed from soaps by extraction with water and repeated washings.

A study of this chart shows that in the first tube soaps were not present in sufficient quantity to inhibit the action of the trypsin, but that digestion in a similar fraction treated with iodin was as great at the end of twenty-four hours as that obtained in the first tube after ninety-six hours. In the third tube the soaps had evidently been removed to a large extent, though digestion in the last seventy-two hours was not equal to that observed in the iodized fraction.

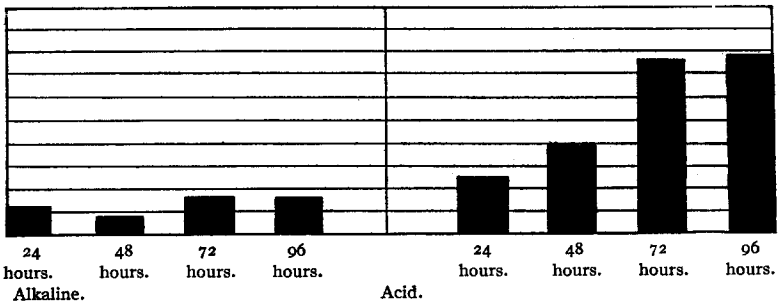
CASEOUS PNEUMONIA.

The affected areas in the caseous lungs were freed as much as possible from the less involved lung tissue, cut up in a meat machine, and dried. When dry the material was ground to a fine powder in a mortar. We anticipated somewhat different results in these experiments from those obtained with caseous material from lymph glands, owing to the difficulty of freeing the caseous material from the inflammatory exudate. In the former case there is usually no inflammatory exudate and therefore no extraneous ferments, while in the material from caseous pneumonia these ferments must be considered.

Several specimens of this kind were examined, and practically similar results were obtained with all. As in the experiments conducted with the caseous matter of lymph glands, the emulsion was made in the proportion of one gram of the dried material to one hundred cubic centimeters of water.

In the first experiment we wished to determine the extent of autolysis. Two flasks containing twenty-five cubic centimeters each of the emulsion were incubated for several days at 37° C. In order to determine the type of ferments present, the contents of one of

the flasks was made slightly acid in reaction, and the other slightly alkaline. Text-figure 3 shows the result of this experiment. In studying this chart it should be remembered that soaps are dissociated in an acid reaction, and are therefore inactive as inhibiting agents. Each line on the chart represents 0.1 of a milligram of nitrogen, and the black columns indicate the incoagulable nitrogen in



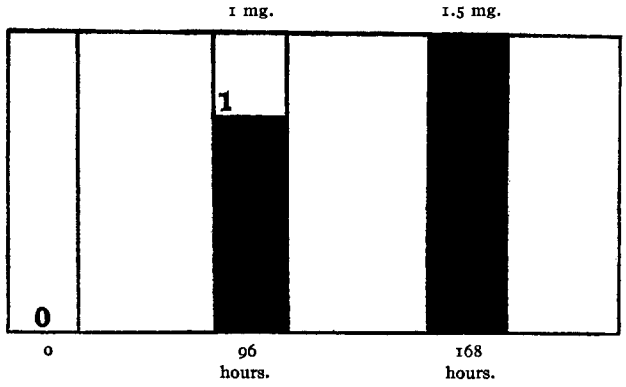
TEXT-FIG. 3. Autolysis of caseous material in acid and alkaline reactions.

two cubic centimeters of the emulsion. The chart shows the differences obtained in the presence of acids and alkalies. Inhibiting substances were present in sufficient quantity to prevent the action of ferments active in an alkaline reaction, but they were not effective when the reaction was made acid.

In some of our preliminary experiments we observed that autolysis occurred in certain of the emulsions which had been made slightly alkaline, and we were at a loss for the proper explanation as soaps were known to be present. We found later that in practically all such instances the reaction of the emulsion had become acid, thus throwing out the soaps and permitting the action of ferments active in an acid reaction. In the last experiment it was evident that ferments active in an acid reaction were present, but that those active in an alkaline reaction were absent or their activity was suppressed by the soaps. In the next experiment we wished to determine if ferments active in an alkaline reaction were also present in the caseous material.

The emulsion was treated with an excess of alcohol which had been made slightly acid. By this means we hoped to precipitate the ferments uninjured and at the same time to remove the soaps, or

rather the fatty acids, which, treated in this manner, are soluble in alcohol. The precipitate was removed by centrifugalization, washed with alcohol and ether, and dried. It was then brought up to the original volume with water, made slightly alkaline, and placed in the incubator for seven days. The results of this experiment are seen in text-figure 4. The columns represent the amount of incoag-



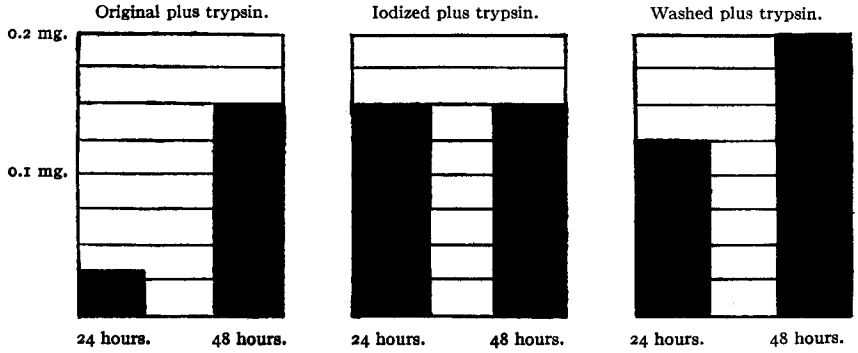
TEXT-FIG. 4. Autolysis of alcohol precipitate.

ulable nitrogen in two cubic centimeters of the mixture. The chart shows that ferments active in an alkaline reaction were present in the original material, but that their activity was inhibited by the soaps. When these were removed by the acid alcohol, the caseous matter underwent autolysis. It is unlikely that these tissues become acid during life, and so the lack of autolysis is easily explained.

Soaps were present in smaller amounts than in the caseous matter of lymph glands. This may be due to the fact that the rapidity of the process did not permit an accumulation of the soaps such as probably occurs in the chronic tuberculous lymph glands. Text-figure 3 shows that autolysis did not occur when the original emulsion was made slightly alkaline, though in other experiments in which the soaps were first removed, we demonstrated the presence of ferments active in an alkaline reaction. These results indicate a slight excess of soaps over the amount needed to prevent autolysis. This view is confirmed in the next experiment in which it is shown that with an increase of ferment autolysis ensues.

In our experiments with the caseous matter of lymph glands we

showed that previous treatment of the emulsion with iodin was effective in aiding the action of trypsin. We now made similar experiments with the pneumonic material, using the same technique. Text-figure 5 shows the results obtained. Two controls were used.



TEXT-FIG. 5. Effect of iodin on ferment-inhibiting substances in caseous pneumonia.

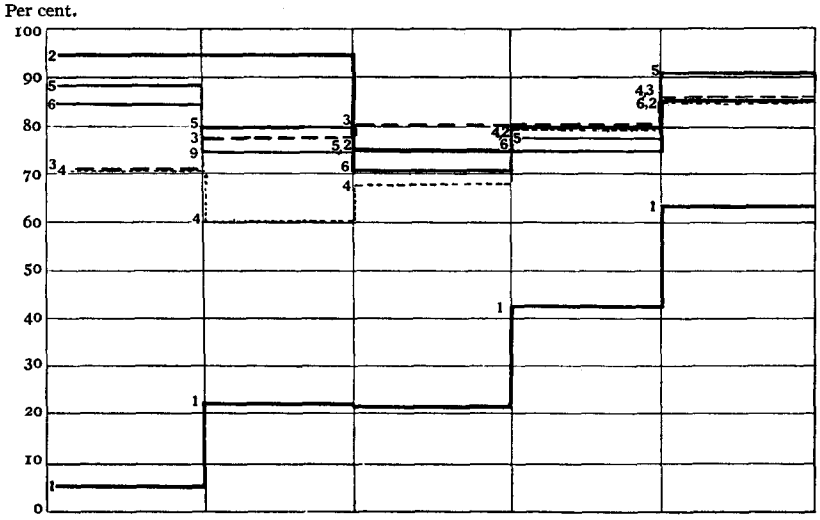
One control consisted of the untreated emulsion with trypsin, and the other of the washed precipitate with trypsin. This last control was used to see if all the soaps could be removed from the precipitate by repeated washings with water.

Here again we have evidence that previous treatment of the emulsion with iodin increased the activity of the trypsin. The amount of incoagulable nitrogen obtained after twenty-four hours' incubation of the iodized emulsion was much greater than that observed in the untreated fraction, and also greater than that obtained with the washed precipitate. It is probable that the washed precipitate was not entirely free of soaps. The amount of digestion observed after forty-eight hours in the untreated emulsion indicates that soaps were not present in large amounts. In a previous paper (9) we showed that soaps were able to inhibit completely the action of enzymes only in certain proportions, and that digestion occurred when enzymes were present in excess of this proportion. This is probably the explanation in the present instance, as the results show that soaps are present in comparatively small amounts in these pneumonic areas (text-figure 6).

In addition to the extractives of the tubercle bacilli which we have

been discussing, lipoids derived from dead cells are also present in caseous matter. Neumann (10) and others state that an alkaline solution of lecithin inhibits tryptic activity, but Meyer (11), using a 1 per cent. solution, was unable to confirm these observations.

In view of the uncertainty of the action of the lipoids we made a few experiments in order to determine their influence on enzyme activity. For these experiments lecithin, cuorin, and cholesterol were used. The solutions were prepared by mixing one gram of the lipoid with 100 cubic centimeters of a 1 per cent. solution of an inactive preparation of sodium oleate. This sodium oleate prepara-



1 = soaps of tubercle bacilli.
2 = sodium oleate (saturated).
3 = soaps of tubercle bacilli (from waxes).
4 = lecithin.
5 = cuorin.
6 = cholesterol.

TEXT-FIG. 6. Effect of soaps of tubercle bacilli and lipoids on tryptic digestion.

tion was one in which the acid had become saturated, thereby rendering it inactive as an inhibiting agent. By suspending the lipoids in an inactive soap solution we hoped to duplicate the physical condition present in the control containing the soap prepared from tubercle bacilli. Text-figure 6 gives the results obtained in one of the experiments.

In addition to the preparations mentioned above, there was also tested in this experiment a soap prepared by saponifying the acetone-insoluble fraction of the extractives of the tubercle bacilli. This soap is apparently much less active than that prepared from the acetone-soluble fraction.

All the lipoids when tested in this manner appear to have some inhibiting action, particularly lecithin which is the most active. The experiment indicates that lipoids in the tissues, particularly in the presence of soaps, may aid in preventing autolysis, but their activity is much less than that demonstrated for the soaps.

DISCUSSION.

We believe that we are now in a position to understand more clearly the processes leading to caseation in tuberculosis. With the lodging of the bacilli there is death of some of the cells due to the action of the toxins, and a proliferation of the neighboring cells. Under other conditions the death of a small number of cells would be followed at once by their removal, either through the action of phagocytes or by autolysis; but here the phagocytes are absent, and the action of the enzymes is prevented by the presence of the soaps of the unsaturated fatty acids derived from the tubercle bacilli. The anemia caused by the occlusion of the blood vessels plays an important part, as the lack of fluid prevents the dilution and subsequent absorption of the inhibiting substances. As the local process spreads slowly, the reaction at the periphery of the area prevents the entrance of fluids, and thus the caseous area gradually increases in size as the bacilli invade and cause the death of the surrounding tissues.

In conditions such as those present in caseous pneumonia, there is primarily an exudate composed of desquamated cells, leucocytes, and fibrin, which soon becomes caseous. Before caseation is complete in the zone of gelatinous pneumonia, there is not the anemia due to occlusion of vessels that is seen in the more chronic processes, and we must conclude that persistence of caseation is due to substances inhibiting the action of the ferments present in the inflammatory exudate. An important factor in the acute process under discussion is the immense number of bacilli present. Under these conditions

it is not difficult to imagine a corresponding increase in the amount of inhibiting substances, an increase sufficient to neutralize the ferments liberated by the disintegration of leucocytes and other cells. Caseous areas may persist for a long time without undergoing absorption. The rapid softening of these areas subsequent to secondary infections is probably due not only to the entrance of fresh ferments, but also to the fluid accompanying these conditions, the fluid acting as a diluent and aiding in the removal of the inhibiting substances. Soaps if present must exist in very minute quantities in the bacilli. They are probably formed from the neutral fat of the bacilli. The lipases hydrolyze the fats, and the acids are then saponified. Klotz (12) and Bergell (13) have demonstrated the presence of lipases in tuberculous pus and lymphocytes, while Wells and Corper (14) found the ferment present in the tubercle bacilli.

According to Sata (15), most of the fat present in caseous matter is found at the periphery of the area. This may be due either to a wandering in from the outside, or, more probably, to synthesis from the fatty acids liberated during the disintegration of the cells and of the tubercle bacilli. Our experiments indicate that soaps of the unsaturated fatty acids obtained from tubercle bacilli are the active agents in preventing autolysis, and the identification of these acids will probably aid in determining the origin of the neutral fat found in caseous areas.

In our paper on the ferment-inhibiting substances present in tubercle bacilli (16) we reported that iodin combined with the unsaturated fatty acids obtained from the bacilli and neutralized their ferment-inhibiting properties. We also emphasized the importance of this observation in connection with a specific chemotherapeutic agent for tuberculosis. In our present work we have shown that iodin exerts a similar action on the ferment-inhibiting substances present in caseous matter. Thus the iodin may serve another purpose by aiding in bringing about solution and absorption of the caseous matter, and exposing the bacilli, which otherwise are more or less inaccessible, to the influence of the therapeutic agent.

It is a well known clinical observation that iodides cause tubercle bacilli to appear in the sputum of patients with pulmonary tuberculosis, though they were previously absent. This phenomenon is

probably due to the neutralization of the action of the ferment-inhibiting substances by the iodin. Subsequent to this neutralization, ferment action ensues, with liberation of the bacilli and their appearance in the sputum.

The autolysis that occurs in the center of large anemic infarcts, and the lack of autolysis at the periphery, can be explained in the same manner. At the periphery of the infarct the fatty acids which are liberated following the death of the cells are saponified by the alkalies present in the tissues, and by those coming from the surrounding fluids. The soaps thus formed inhibit proteolysis until the phagocytes, invading the periphery, open up channels through which sufficient fluid can enter to wash out or dilute the soaps so that autolysis can proceed. The phagocytes of course also take part in the removal of the dead tissues. In the center of the infarct the alkalies are soon used up and the tissues then become acid in reaction on account of the excess of fatty acids. Wiener (17) found that autolytic enzymes act best in an acid medium, and this is to be expected as soaps are inactive as inhibiting agents under these conditions.

CONCLUSIONS.

1. Caseous matter obtained from lymph glands which have not become secondarily infected contains substances which inhibit enzyme activity. These substances consist chiefly of soaps of the unsaturated fatty acids.

2. The inhibiting substances are present in relatively smaller amounts when the caseous matter has become secondarily infected. This is probably due to the dilution and washing out of the soaps.

3. Ferments are either entirely absent or present in very small amounts, unless the caseous matter has become secondarily infected.

4. Caseous material from the lungs contains smaller amounts of the inhibiting substances. This may be due to the acuteness of the process, which does not permit an accumulation of the soaps, or to the binding of the soaps with the ferments.

5. Ferments are present in caseous pneumonia. In the whole emulsion the ferments are less active in an alkaline than in an acid reaction; but removal of the soaps shows that those active in an alkaline reaction are also present in considerable amounts.

6. The previous treatment with iodin of caseous matter from both lymph glands and lungs increases the action of the trypsin.

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