SOAPS AS FERMENT-INHIBITING AGENTS.

STUDIES ON FERMENT ACTION. X.*

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The use of the antitryptic index of the blood as a clinical test, particularly in cancer, and the pathological conditions in which ferment action appears to be held in abeyance, especially in caseation in tuberculosis and syphilis, in anemic infarcts, and in the exudate in lobar pneumonia, make the study of ferment-inhibiting substances of great importance. Many substances are considered to have the property of inhibiting the activity of enzymes, but in this paper we shall refer chiefly to the proteolytic enzymes acting in an alkaline medium.

Trypsin and leucoprotease lose their activity when heated at temperatures above 60° C., though according to Salkowski (1) trypsin when dry can be heated to 160° C. without being destroyed. Strong acids and alkalies quickly destroy trypsin, but Chittenden and Cummins (2) found it much more resistant to these agents in the presence of protein. Kudo (3) states that all the mineral acids are active in this respect, sulphuric acid being active in a dilution of one in a thousand, while the organic acids are much less so. Weiss (4) reports that while 0.05 per cent. sodium chloride increases the activity of trypsin, 10 per cent. decreases it. Most of the writers who have investigated the subject find that chloroform, toluol, and thymol inhibit the action of trypsin to a slight degree, but the necessity of a preservative in their experiments caused most workers to ignore this slight inhibition. Bayliss (5) and Abderhalden and Gigon (6) found that some of the products of tryptic activity possess the property of inhibiting the action of the ferment, the free amino acids being more active than the polypeptids. It has been known for several years that blood serum is capable of inhibiting the proteolytic power of trypsin, but in spite of the work of Camus and Gley (7), Charrin and Levaditi (8), Landsteiner (9), and others, we are still ignorant concerning its nature and significance.

In the course of our studies on ferment action we observed that soaps of certain fatty acids were capable of inhibiting the action of trypsin and leucoprotease. This observation seemed to be of importance, as soaps of a similar nature are present in the body, and the possibility of their acting in the same manner

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caused us to make a more careful study of the general phenomena relating to the inhibition of ferment activity by these substances.

Neumann (10), while studying the influence of a fat-rich diet on the digestion of infants, observed that sodium oleate inhibited the action of trypsin. For most of his work he used the Fuld-Gross technique. In some of our preliminary experiments we used the same technique, but soon discarded it in favor of the one which will be described later. According to the Fuld-Gross method the mixture of ferment and substrate is incubated for two hours and then made slightly acid by adding a few drops of a solution containing 5 per cent. acetic acid in 50 per cent. alcohol. The amount of digestion is determined by the degree of cloudiness, the more complete the digestion the clearer the contents of the tube after acidifying. Subsequent experience convinced us of the unreliability of this method, particularly when dealing with lipoidal substances. The acidifying not only coagulates the undigested protein, but also causes the lipoidal substances, particularly the fatty acids, to separate as flocculi, a condition which makes it impossible to determine the extent of proteolysis. Neumann added an excess of alcohol to cause a solution of the precipitated acids and thus solved one difficulty; but he added another by diluting the protein to such a degree that it became a problem to determine the extent of proteolysis.

Two ferments, trypsin and leucoprotease, were used in our work. The trypsin was obtained by extracting commercial pancreatin with N/50 sodium carbonate. After extraction for twenty-four hours, the solution was filtered, and the trypsin in the filtrate precipitated by the addition of nine volumes of alcohol to which sufficient acetic acid had been added to make the whole mixture slightly acid to litmus. This extraction and precipitation was repeated three times. The final product was much stronger than the original and the amount needed to cause complete digestion in the control tubes did not contain sufficient nitrogen to give a definite color when Nesslerized. The leucoprotease was prepared from human pus in the same manner, but the amount required to cause complete digestion in the control tubes contained more nitrogen than the concentrated trypsin. In each experiment the amount of nitrogen was determined in the control tubes and proper correction made in calculating the amount of total incoagulable nitrogen.

Edestin was used as a substrate for several experiments, but later it was discontinued in favor of a 1 per cent. casein solution, the latter being much more soluble in an alkaline medium, and in addition it was noted in precipitating the coagulable protein that a slight excess of acid caused the edestin precipitate to redissolve. After being incubated for the necessary time, the contents of the tubes

were acidified with a mixture containing 10 per cent. glacial acetic acid and 20 per cent. sodium chloride, and the tubes placed in boiling water for five to ten minutes. The mixtures were then filtered through kaolin and the incoagulable nitrogen was determined by the method recommended by Folin (11). The amount of nitrogen found in the control tubes was subtracted from each of the others and the results were given in percentages of total digestion.

The time allowed for incubation was not the same in all experiments. In our preliminary work we found that the concentrated trypsin, prepared as described above, deteriorated rapidly when once dissolved. For this reason the trypsin solution was prepared immediately before being used, one cubic centimeter representing 0.002 of a gram of trypsin. Several tubes containing only casein and trypsin were placed in the incubator at the same time as the tubes or flasks containing the mixtures of soaps. One of the tubes containing casein and trypsin was removed after one hour and tested. After that the other tubes were removed at intervals of fifteen minutes, also tested, and as soon as digestion was found to be complete the remainder were immediately removed, acidified, and boiled.

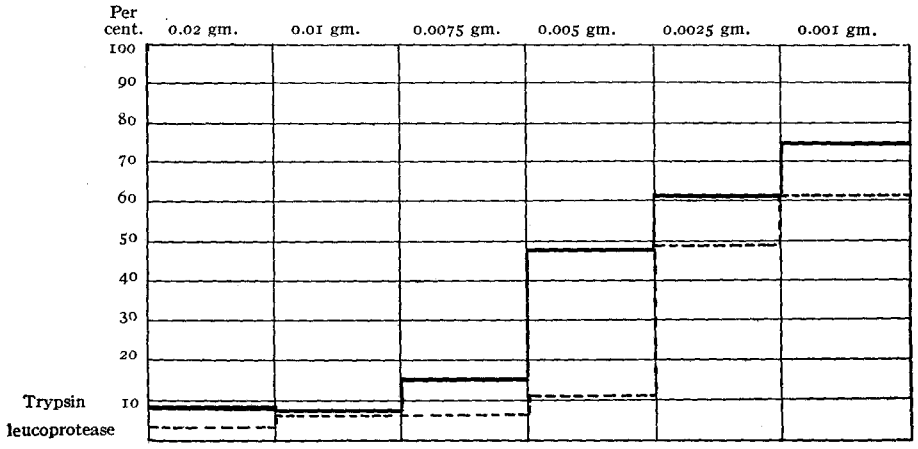
The protocols are not given for each experiment as the charts present the results more clearly. Table I gives the number of controls used in each experiment, and also the amount of nitrogen obtained by complete digestion and as a result of the inhibiting action of the soaps.

In most instances we did not know the identity of the acids used in the preparation of the soaps, but the isolation and identification of these with further work on their ferment-inhibiting action is now under way. The three supposedly pure soaps were Kahlbaum's preparations, and consisted of sodium oleate, sodium palmitate, and sodium stearate. On examination we found that the sodium oleate had become saturated, making it useless as an oleate, though it was used in several experiments as a representative of the saturated fatty acids.

The majority of the soaps were obtained by saponifying linseed, olive, cod-liver, hempseed, and castor oils, as these oils are known to contain a large number of unsaturated fatty acids. The oils were saponified with alcoholic potash, and the soaps extracted re-

peatedly with petroleum ether. The acids were then liberated by hydrochloric acid, taken up in ether, the ether evaporated, and the acids again saponified. This process was repeated and the acids were preserved pure until needed. The soaps used in the work were rarely more than two or three days old.

Later when it was found that the unsaturated fatty acids were the active inhibiting agents, the lead soap-ether method of obtaining these in higher concentrations was used. The separation of the saturated and unsaturated fatty acids by this method is not complete, but is sufficiently so to enable us to show marked difference between the action of the ether-soluble and insoluble fractions. In

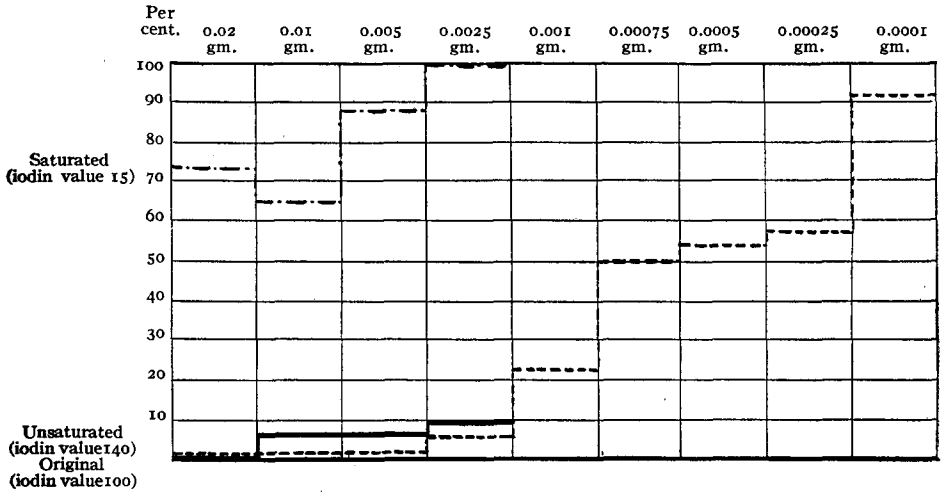


TEXT-FIG. 1. Effect of sodium oleate on tryptic and leucoproteolytic digestion.

nearly every instance the iodin value of the preparations was determined before they were used.

One of the first soaps investigated was that prepared from olive oil. Its influence on leucoprotease and trypsin is shown in text-figure 1. In this experiment the soap and ferment were mixed, incubated for thirty minutes at 37° C., and the casein was then added. This procedure was adopted in all the subsequent experiments. Text-figure 1 shows that even 0.001 of a gram of the soap is sufficient to affect materially the activity of both ferments. In this and in subsequent charts the control tubes showing 100 per cent. of digestion are not given.

Efforts were then made to determine if the inhibiting action displayed by these soaps was due to the saturated or unsaturated fatty acids present. Soaps from nearly all the oils were tested in a similar manner, but as uniform results were obtained, we shall give only one example. Text-figure 2 shows the results obtained with



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

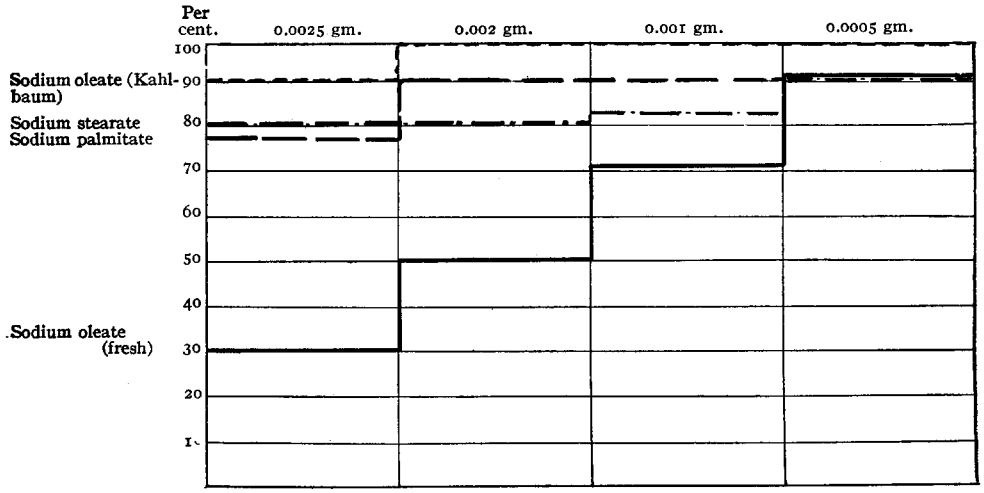
the soap prepared from linseed oil. The ether-lead soap method was used in separating the groups of acids. The chart indicates that the unsaturated fatty acids are the active inhibiting agents. It is well known that the ether-lead soap method does not give a complete separation of the saturated and unsaturated fatty acids, and this probably explains why the saturated fraction, the ether-insoluble lead soaps, still retained about one fourth of its inhibiting action, whereas the unsaturated fraction, the ether-soluble lead soap, now caused complete inhibition.

During the progress of the work we obtained three of Kahlbaum's supposedly pure soaps, sodium oleate, sodium palmitate, and sodium stearate. The results obtained with these three soaps and with one prepared by ourselves from olive oil are shown in text-figure 3. The iodin value of the linseed oil soap prepared by us was 90.

Our previous experiments indicated that the soaps of all the unsaturated fatty acids acted as inhibiting agents. We were therefore

surprised to note that the Kahlbaum sodium oleate was inactive. The solution of the problem was soon found, the sodium oleate had no iodin value; in other words, the oleic acid, through age or some other cause, had become saturated. The sodium soaps of palmitic and stearic acids, as we had anticipated, had no influence on either trypsin or leucoprotease.

All our experiments so far indicate that the inhibiting agents present in the soaps are the unsaturated fatty acids. The question therefore naturally presents itself: Can we by a saturation of these



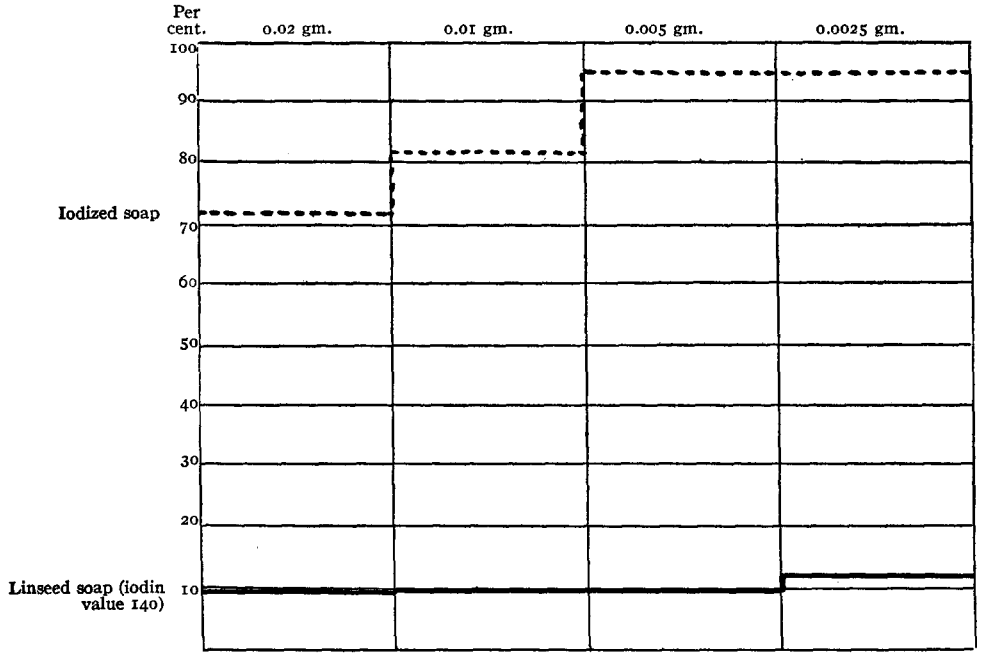
TEXT-FIG. 3. Effect of sodium oleate, sodium stearate, and sodium palmitate on tryptic digestion.

free bonds with iodin, for instance, destroy the inhibiting action and cause them to act like the soaps prepared with stearic, palmitic, and other saturated acids?

The results of one of the experiments made to enlighten us on this point are given in text-figure 4. This experiment was conducted with the ether-soluble lead soap fraction. To five cubic centimeters of a 1 per cent. solution of the soap were 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 action of the unsaturated soap before and after it was treated with iodin. Repeated tests had already shown that the small amount of iodin which might still remain would have a slight inhibiting action. This would explain why the soap still had some inhibiting action, though it may also be due to the fact that the acids were not completely saturated. The results of saturating the free carbon bonds are so obvious as to call for no comment, and also explain why the commercial sodium oleate was inactive.

Similar experiments made with sodium soaps of unsaturated fatty acids obtained from other sources indicated that the inhibiting



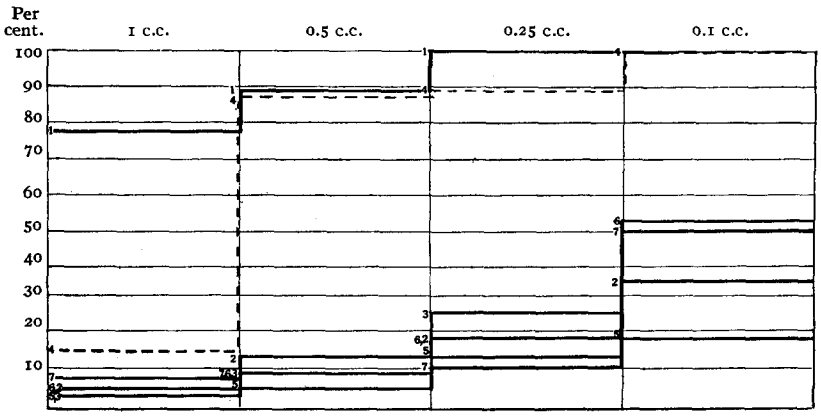
TEXT-FIG. 4. Effect of saturation of unsaturated linseed soaps with iodin.

action was due to the degree of unsaturation and that it could be almost entirely removed by saturation with iodin.

Our next experiments were made to determine whether the activity of the various soaps was dependent upon the number of unsatu-

rated bonds, as determined by their iodin values, or whether certain ones containing an equal number of unsaturated bonds were more active.

In this experiment soaps prepared from linseed oil, hempseed oil, castor oil, olive oil, croton oil, and cod-liver oil were used. After their respective iodin values had been determined, they were made up in such strengths that one cubic centimeter of each had the same iodin value; thus croton oil soap having less than half the value of the linseed oil soap was made up twice as strong. Text-figure 5



1 = saturated linseed soap.	Iodin value 15.	Solution 5.0	per cent.
2 = sodium oleate.	Iodin value 53.	Solution 1.9	per cent.
3 = croton soap.	Iodin value 55.	Solution 1.8	per cent.
4 = castor oil soap.	Iodin value 60.	Solution 1.7	per cent.
5 = hempseed soap.	Iodin value 100.	Solution 1.0	per cent.
6 = cod-liver oil soap.	Iodin value 90.	Solution 1.1	per cent.
7 = linseed soap.	Iodin value 135.	Solution 0.74	per cent.

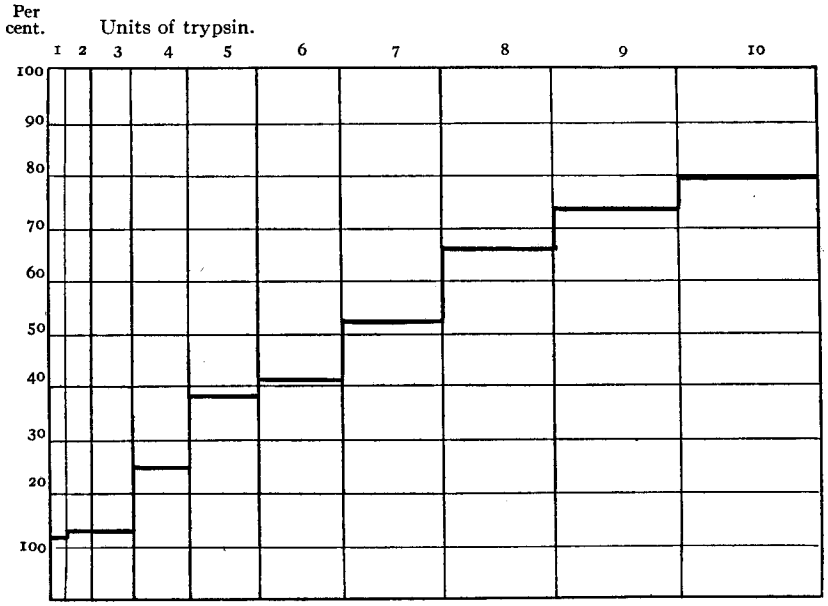
TEXT-FIG. 5. Relation of iodin value to inhibiting action of soaps on tryptic activity.

gives the results of this experiment. The lines shown on the chart indicate, with one exception, that the activity of the soaps is in proportion to their iodin values.

Here we have the first indication that the unsaturated fatty acids are not equally active as inhibiting agents, for the soap prepared from castor oil appears to be considerably less active. The inhibition obtained with one cubic centimeter was almost as great as that noted with the other soaps, but subsequent dilution proved it to be

much less active. With the exception of that prepared from linseed oil, the soaps used in this experiment were not obtained by the ether-lead soap method, and so their iodin values were low. It is possible that some of the unsaturated fatty acids are much more active than others in this respect. In fact, a part of our recent work indicates that those obtained from a certain source are much more active than any of those we have just discussed.

In the preceding experiments it has been demonstrated that 0.005 of a gram of the linseed oil soap was sufficient to prevent the action of the standard amount of trypsin. It was also found, in the standard proportions of ferment and substrate used, that the inhibiting action was still present in tubes containing much smaller amounts. In fact complete digestion was first obtained in the tube containing



TEXT-FIG. 6. Effect of increasing amounts of trypsin with single unit of soap.

0.0001 of a gram. In our next experiment we wished to find out if the smallest amount of soap producing nearly complete inhibition with the standard amounts of trypsin and casein would have any influence on larger amounts of the ferment. Text-figure 6 shows

the influence of the soap on increasing amounts of the ferment. 0.1 of a cubic centimeter of the ferment solution caused complete digestion in the control tube. The demonstration that such a small amount of soap, 0.005 of a gram, was able to affect materially the action of ten times the standard amount of ferment, indicates the great activity of these substances as inhibiting agents.

In the previous experiments we have shown that the unsaturated fatty acids inhibit proteolysis in an alkaline medium and that the degree of inhibition bears a close relation to their iodin values, but these experiments do not give us much information as to how the inhibition is effected. Is it due to a direct and permanent binding of the ferment with the unsaturated fatty acids, or to simple inhibition of ferment action, the ferment itself not being destroyed? If there is a true binding it would be difficult to extract the ferment after incubating with soap, but it might be done if the inhibition were due to some other cause.

Our next experiment was planned with the hope that some information might be obtained as to the nature of the inhibiting action. In this experiment the soaps of the saturated and unsaturated acids separated by the ether-lead soap method were mixed with the ferment and incubated for thirty minutes. The mixtures were then made slightly acid with hydrochloric acid and extracted with ether. After having removed the ether, the solution was made slightly alkaline, the casein added and again incubated. One control tube of ferment without soap was treated in the same manner so as to rule out the effect of the acid and ether. In this experiment we wished to find out if the soap simply inhibited the action of the ferment or destroyed it. Table I gives the results of this experiment.

TABLE I.

	Trypsin.	1 per cent. unsaturated linseed soap, iodin value 130.	1 per cent. saturated linseed soap, iodin value 15.	Sodium chloride solution.	1 per cent. casein.	Total incoagulable nitrogen.	Digestion.
1	0	0	0	3.0 c.c.	2.0 c.c.	0.04 mg.	2 %
2	0.2 c.c.	0	0	2.8 c.c.	2.0 c.c.	1.66 mg.	100 %
3	0.2 c.c.	0.5 c.c.	0	2.3 c.c.	2.0 c.c.	0.10 mg.	6 %
4	0.2 c.c.	0	0.5 c.c.	2.3 c.c.	2.0 c.c.	1.30 mg.	80 %
5	0.2 c.c.	0	0	2.8 c.c.	2.0 c.c.	1.50 mg.	90 %
6	0.2 c.c.	0.5 c.c.	0	2.3 c.c.	2.0 c.c.	0.20 mg.	12 %
7	0.2 c.c.		0.5 c.c.	2.3 c.c.	2.0 c.c.	1.66 mg.	100 %

The first four tubes in the table are the untreated controls. The first tube shows the percentage of incoagulable nitrogen in the casein alone; the second, the total nitrogen in complete digestion; the third and fourth show the influence of the saturated and unsaturated soaps on tryptic activity. Tubes 5, 6, and 7 show the influence of acid and ether extraction. Tube 5 contained no soap but was treated in the same way as Nos. 6 and 7 to make certain that the method used had not destroyed the ferment. Tube 6 containing the unsaturated soap shows that the ferment action is almost inhibited, while in tube 7 containing the saturated soap there is no inhibition. In other words, incubating the ferment with soaps of the unsaturated fatty acids for thirty minutes causes its destruction, the removal of the acids after incubation not being sufficient to reactivate the ferment, while the ferment present in the tube containing the saturated acid, though treated in the same manner, is not destroyed.

The results obtained in this preliminary study of the action of the soaps prepared from the unsaturated fatty acids suggest that they may play an important part in the body, particularly in certain pathological processes. Whether this inhibition of ferment action is due to a binding of the ferment with the unsaturated carbon atoms, or whether to some physical condition brought about by the presence of the soap, remains to be determined. If due to some physical condition brought about by the presence of the soap, similar results ought to be obtained with the saturated fatty acids, but we have demonstrated that the latter are inactive in this respect. In addition, the experiments conducted with soaps saturated with iodin show that the presence of unsaturated bonds are necessary for the development of this property. Further proof that this action is due to the degree of unsaturation is afforded by text-figure 5, which indicates that the activity of the soaps is proportionate to their iodin value, while the soaps having no iodin value, sodium stearate and sodium palmitate, are inactive.

SUMMARY.

1. Sodium soaps prepared from olive oil, croton oil, cod-liver oil, linseed oil, etc., have the property of inhibiting the action of trypsin and leucoprotease.

2. The activity of these soaps is dependent upon the degree of unsaturation of the fatty acids and is in proportion to their iodin value.

3. Saturation of the acids with a halogen (iodin) causes a loss of this property.

4. Soaps of the saturated fatty acids tested do not have this influence on ferments.

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