THE ACTION OF CERTAIN BACTERIA ON SOME SIMPLE TRI-GLYCERIDES AND NATURAL FATS, AS SHOWN BY NILE-BLUE SULPHATE[1]

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In studies on the metatrophic bacteria, action on carbohydrates and proteins has been given much more attention than action on fats. This has been due, in part, to the inability of many of the common bacteria to attack fats, but the relative inconvenience of determining lipolytic action has been a factor also. It appears that more attention to the action of bacteria on fats should yield information of importance in studies on the deterioration of various foods and also in investigations on the systematic relationships of the organisms.

HISTORICAL

In 1894, Sommaruga studied the lipolytic ability of microorganisms by growing them on solid media in which was dispersed 2.0 per cent olive oil or other fat. No indicator was used, and the disappearance of the fat globules from the region surrounding the growth of the bacteria was accepted as evidence of hydrolysis.

Eijkman (1901) inoculated melted agar and poured it over a thin layer of tallow in a petri dish. Lipolytic organisms formed colonies that caused clear zones in the tallow beneath them.

The simultaneous staining of neutral fats and fatty acids by oxazine dyes was studied by Smith (1908). This investigator pointed out that fat globules contained in the tissues could be stained by basic aniline dyes if they were first changed into fatty

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acids. When an aqueous solution of Nile-blue sulphate was shaken with olein the fat became red, but when shaken with oleic acid the fatty acid became blue or bluish purple, depending on the relative amounts of blue and red substances present in the solution of the dye. The blue color was due to the blue soap resulting from the reaction between the fatty acid and the oxazine base.

Sayer, Rahn and Farrand (1908) detected lipolysis by bacteria with a litmus agar, prepared from sugar-free broth, in which was dispersed a small amount of butter fat. Lipolysis was indicated by a change in the color of the indicator.

Boeminghaus (1920) studied the color reactions of Nile-blue sulphate with palmitin, stearin and olein, their corresponding acids, and certain other derivatives; some natural fats were also used. He emphasized the intense colors secured with oleic acid and its ester combinations (glycerol and cholesterin), the free acid being blue and the combinations red. Palmitic and stearic acids and palmitin and stearin were only slightly colored. In connection with the intense color of oleic acid, compared to other fatty acids, this investigator noted that oleic acid is an unsaturated acid.

Buchanan (1921) pointed out that lipolytic organisms, when grown on a solid medium in which was dispersed a suitable fat or oil, produce a lipase which causes the disappearance of the fat from the immediate vicinity of the growth. Waksman and Davison (1926) emphasized the use of the changes produced in certain indicators by the freed fatty acids to detect fat hydrolysis through the action of organisms.

The detection of fat hydrolysis by bacteria was investigated by Turner (1927). He used a medium composed of 1000 ml. of sugar-free meat digest fluid, 5 grams of di-basic sodium phosphate and 30 grams of agar, the reaction of the medium being adjusted to a final pH of 7.6. After autoclaving, Nile-blue sulphate was added and the medium tubed and heated in an Arnold. A sterile fat emulsion was added to the melted and cooled medium. Turner (1929) compared the relative merits of various methods for the determination of fat hydrolysis by microörganisms and concluded that the Nile-blue sulphate medium gave remarkable sharpness of differentiation. He used various simple tri-glycerides

in the medium and found that tri-butyrin inhibited growth of the two test cultures. Nile-blue sulphate, in concentrations of 1:8000, inhibited the growth of a number of organisms; the flooding of plates with Nile-blue sulphate solution after incubation was suggested for certain purposes. Turner considered that a good differential plating medium, without dye, for the detection of lipolytic bacteria should result from either an emulsion of fat made up of short-chained tri-glycerides or from the use of bile in the medium along with an oil like cottonseed oil.

Kaufmann and Lehmann (1926), studied the action of various stains, including Nile-blue sulphate, on different organic acids, esters and other materials. They noted the striking effect of Nile-blue sulphate on unsaturated fatty acids and unsaturated tri-glycerides but found that certain compounds without double bonds were also stained.

Rettie (1931) considered that Nile-blue sulphate contains two coloring reagents, the blue which is soluble in the fatty acids and the pink which is soluble in the fats; the latter he called Nile-pink.

Turner's technique was somewhat modified by Hussong (1932). Hussong used beef infusion agar with a pH of 6.8 to 7.0. To this he added Nile-blue sulphate (as an alcoholic solution) in the ratio of 1:10,000 and fat in the proportion of 1:200. The fat was emulsified in 0.5 per cent agar and was sterilized before it was added to the medium. Cultures were streaked on the surface of the medium. A change in the color of the fat globules was accepted as evidence of hydrolysis.

Berry (1933) worked with the test originated by Carnot and Mauban (1918) for the detection of microbial lipase. The organisms were grown on a solid medium having fat dispersed in it, and when good growth had occurred the plates were flooded with saturated copper sulphate solution. If hydrolysis had taken place, the freed fatty acids reacted with the copper sulphate to give an insoluble blue soap.

STATEMENT OF PROBLEM

The work herein reported was undertaken (*a*) to determine the action of Nile-blue sulphate on various simple tri-glycerides and the corresponding fatty acids and on some natural and hydro-

genated fats, (*b*) to study certain factors affecting the detection of lipolysis by bacteria, using Nile-blue sulphate and (*c*) to determine the susceptibility of various simple tri-glycerides and natural and hydrogenated fats to the action of lipolytic bacteria. All of the trials were carried out in agar media.

GENERAL ACTION OF NILE-BLUE SULPHATE ON FAT

The general action of Nile-blue sulphate in the staining of fat was shown by the investigations of Thorpe (1907) which followed observations by Smith and White that certain blue coloring materials of the oxazine series, when used as a stain for sections containing neutral fat, possessed the property of coloring fatty matter red, while protein matter was stained blue. Smith and White had noted that the red stain could be extracted by xylene. Thorpe found that the red coloring matter could not be secured from the solid dye, but that it was apparently formed when the dye was dissolved in water. By treating the dye dissolved in water with sulphuric acid the formation of the red dye readily took place and it could be extracted with xylene; the production of the red dye was increased by heating the mixture on a water bath. In the preparation of certain fat stains the dye is boiled with acid to intensify the staining of fat. Nile-blue sulphate is a sulphate of diethylaminophenonaphthoxazine and the red dye the corresponding oxazone.

METHODS

The medium ordinarily used for studying the action of Nile-blue sulphate on simple tri-glycerides, fatty acids and natural and hydrogenated fats, and also for investigating the action of bacteria on simple tri-glycerides and fats, was beef-infusion agar adjusted to a pH of 6.8 to 7.0. A 0.1 per cent aqueous solution of Nile-blue sulphate was added to the agar, in the proportion of 10 to 100 ml. of medium, and the agar then put into tubes or flasks and sterilized. Lower concentrations of Nile-blue sulphate were also used with satisfactory results, but the colors were less intense.

The emulsions of natural and hydrogenated fats were prepared as follows: The fat to be used was filtered with a hot water funnel

and added to a melted 0.5 per cent agar solution in the proportion of 10 ml. of fat to 90 ml. of the solution. The mixture was sterilized at 15 pounds for twenty-five minutes, allowed to cool until it was solidified and then vigorously shaken to secure an emulsion of the fat. The fat emulsion was stored in this condition and just before use was heated to a temperature that would give a soft jelly-like mass which could be easily transferred with a pipette.

When plates were to be poured, agar containing Nile-blue sulphate was melted and the fat emulsion added to the hot agar in the proportion of 1 ml. of the emulsion to 20 ml. of the agar. After the dye had been added to the agar the medium was allowed to remain hot for a few minutes before it was poured.

The liquid tri-glycerides and fatty acids were dispersed in agar containing Nile-blue sulphate in the same general manner as the natural and hydrogenated fats, excepting that smaller quantities were used because of the cost. The tri-glycerides and the fatty acids which are solid at 21°C. were dispersed as follows: Agar containing Nile-blue sulphate was added to the plates and kept hot over a low Bunsen flame, while a small amount of the solid tri-glyceride or fatty acid was added and vigorously stirred into it. The agitation was continued until the fat or fatty acid had solidified in small globules or masses.

In the study of the effect of various bacteria on the simple tri-glycerides and natural and hydrogenated fats, the plates poured with the materials were left at room temperature until the surface of the medium was dry (at least twelve hours) to prevent an abnormal spreading of the bacterial colonies. Several organisms were inoculated on each plate, using a small loop-full of a forty-eight-hour litmus milk culture of each organism to be studied. The plates were inverted, incubated at 21°C. and examined frequently for evidence of lipolysis.

All the examinations for color changes and for disappearance of the globules were made with a hand lens or a wide field binocular.

The tri-olein was prepared by Dr. Fraenkel and Dr. Landau, Berlin, while the other simple tri-glycerides were secured from the Eastman Kodak Company. Some of the fatty acids were ob-

tained from the Eastman Kodak Company, and the others from Merck and Company. The natural and hydrogenated fats were secured locally.

RESULTS OBTAINED

Action of Nile-blue sulphate on various simple tri-glycerides, fatty acids and natural and hydrogenated fats

The action of Nile-blue sulphate on various simple tri-glycerides, fatty acids and natural and hydrogenated fats was studied by pouring plates with agar containing Nile-blue sulphate, as outlined under Methods. The plates were held at approximately 21°C. and examined frequently. In a number of trials, an agar containing no added nutrients was employed in addition to beef-infusion agar; the results were the same with the two media.

Action on simple tri-glycerides. The simple tri-glycerides used were tri-acetin, tri-propionin, tri-butyrin, tri-caproin, tri-caprylin, tri-caprin, tri-laurin, tri-myristin, tri-palmitin, tri-olein and tri-stearin.

Tri-acetin, in the concentrations used, was completely soluble in the medium. The globules of tri-propionin, tri-butyrin, tri-caproin and tri-caprylin were bright red in color. The dispersed masses of tri-caprin, tri-laurin, tri-myristin, tri-palmitin and tri-stearin were solid and colored red to a degree which decreased rapidly with the increase in the melting points, until very little red color was present. A rather uniform distribution of fairly round solid globules was obtained with tri-caprin and tri-laurin, while with tri-myristin, tri-palmitin and tri-stearin a dispersion of somewhat flaky, irregularly shaped masses was secured; the color of these irregular masses ranged from a faintly purplish red to a faintly reddish purple and, occasionally, to a bluish white. The dispersed globules of tri-olein were very uniformly bright red and regular in size.

Action on fatty acids. The fatty acids employed were acetic, propionic, butyric, caproic, caprylic, capric, lauric, myristic, palmitic, oleic and stearic.

Acetic, propionic and butyric acids were soluble in the media in the concentrations used and, with the occasional exception of butyric acid, they did not affect the appearance; butyric acid sometimes caused a turbidity in the media. The dispersed globules of caproic and caprylic acids became distinctly blue and seemed to absorb the dye from the surrounding medium uniformly. The dispersed, solid, disc-like globules of capric and lauric acids ranged in color from a blue that was darker than the surrounding medium to one that was much lighter. The masses of myristic, palmitic and stearic acids were very lightly and not uniformly stained, the stainability seeming to depend on the shape and size of the mass. The globules of oleic acid were always a clear blue that was much darker than the surrounding media.

Action on natural and hydrogenated fats. The natural fats used were beef tallow, butter fat, cocoanut oil, corn oil, cottonseed oil, lard, linseed oil and olive oil. Dispersed portions of all of these materials became distinctly red when added to media containing Nile-blue sulphate. With linseed oil and olive oil the globules were smaller than with the other materials and, presumably because of the size, did not appear as red.

Cottonseed oil and lard that had been commercially hydrogenated gave essentially the same changes with Nile-blue sulphate as the unhydrogenated fats.

Rate of color change when various materials were added to agar containing Nile-blue sulphate. When simple tri-glycerides, insoluble fatty acids or natural fats were added to agar containing Nile-blue sulphate the usual color change occurred very quickly; the time required seemed to be decreased by temperatures considerably above room temperature. When an unemulsified fat was added to hot agar containing Nile-blue sulphate and the agar shaken vigorously for thirty seconds the fat was conspicuously pink by the time it had collected at the surface. Fat which had been sterilized a number of times showed a less rapid and less definite color change with Nile-blue sulphate than fat which had been sterilized only once.

Factors affecting the detection of lipolysis by bacteria, using Nile-blue sulphate

Comparison of the disappearance of globules of simple tri-glycerides and the color changes in them by Nile-blue sulphate. A comparison of the disappearance of globules of simple tri-glycerides and the color changes in them by Nile-blue sulphate for the detection of lipolysis by bacteria was made with 100 miscellaneous cultures. The organisms were isolated from a variety of sources; most of them were lipolytic, but a few non-lipolytic organisms were included as controls. Comparisons could be made best with tri-caproin and tri-caprylin, since the acids of the lower tri-glycerides were completely soluble in the medium in the concentrations used, and the acids of the higher tri-glycerides were insoluble, or only very slightly soluble, but comparisons were also possible with tri-caprin.

With tri-caproin and tri-caprylin, hydrolysis was detected more readily by the disappearance of globules than by the development of a blue color, while with tri-caprin the reverse was true. It appears that this variation is due to the solubility of the acid freed by hydrolysis; if the acid is comparatively soluble its rapid diffusion results in less blue color than if the acid is only very slightly soluble. The best agreement between the two changes was secured with tri-caprin; capric acid was apparently sufficiently soluble so that the disappearance of globules was usually detected, and at the same time sufficiently insoluble so that the blue color was observed. With certain tri-glycerides there was a variation in the results obtained with different organisms so that some organisms caused a conspicuous disappearance of the globules while others caused a conspicuous development of a blue color. Presumably, variations in the action of the different organisms on the primary hydrolytic products influence this reaction.

It should be noted that when natural fats were dispersed in the agar there was no indication of a disappearance of the globules and, for this reason, the color change was always the basis on which the action of the organisms was determined.

Influence of the method of dispersing the tri-glycerides in the medium. The influence of the method of dispersing the tri-

glycerides in the medium on the detection of lipolysis by bacteria was studied with tri-butyrin, tri-caproin, tri-caprylin and tri-olein, the simple tri-glycerides that appear to be most useful in investigating the action of bacteria. These materials were added to melted agar as an emulsion in 0.5 per cent agar and also directly. In each case the medium was then shaken and poured into plates. When the tri-glycerides were emulsified in the agar a more uniform dispersion in the plate was secured than when they were added directly. The comparisons of the action of bacteria on the tri-glycerides dispersed with the two methods showed that the same results were secured with the two procedures.

With the natural fats it was possible to prepare reasonably satisfactory plates by adding the fat directly to the melted agar containing the Nile-blue sulphate, but the dispersion was not as uniform as when the fat was emulsified in 0.5 per cent agar, and there was a tendency for the globules to rise to the surface of the medium and to form masses which interfered with the growth of the organisms.

Influence of the pH of the medium. The effect of the pH on the detection of lipolysis by bacteria in beef-infusion agar containing Nile-blue sulphate was studied with tri-butyrin, tri-olein and cottonseed oil, using 26 cultures of bacteria that had been isolated from various sources; most of the organisms were lipolytic. The pH values used were 5.3, 6.7 and 7.8. With tri-olein and cotton-seed oil an organism which hydrolyzed at one pH also hydrolyzed at the others, although with many of the organisms the most alkaline reaction seemed to favor hydrolysis, as evidenced by the rate at which the color change appeared. With tri-butyrin at a pH of 5.3 the development of colonies was much slower than at a pH of 6.7 or 7.8, and some of the organisms were entirely inhibited; however, if growth occurred, the organisms which hydrolyzed at a pH of 6.7 or 7.8 also eventually hydrolyzed at a pH of 5.3.

Susceptibility of various simple tri-glycerides and natural and hydrogenated fats to the action of lipolytic bacteria

The action of bacteria on various simple tri-glycerides and natural and hydrogenated fats, as shown by Nile-blue sulphate,

was studied with a considerable number of cultures. Most of the cultures were isolated from various sources, but a few were secured from culture collections. Many of the organisms isolated had hydrolyzed fat on the plates used for isolation, but a number which had failed to hydrolyze were also included. The action of each organism was tried three or four times on each simple triglyceride and from two to four times on each natural or hydrogenated fat.

TABLE 1

The hydrolysis of some simple tri-glycerides by 119 cultures of bacteria as indicated by Nile-blue sulphate

TRI-GLYCERIDE	PER CENT OF CULTURES SHOWING		
	Hydrolysis	No hydrolysis	Questionable hydrolysis
Tri-propionin	98.3	1.7	0.0
Tri-butyrin	78.2	17.6	4.2
Tri-n-valerin	68.0	28.0	4.0
Tri-iso-valerin	50.0	40.0	10.0
Tri-caproin	53.8	44.5	1.7
Tri-heptylin	36.0	48.0	16.0
Tri-caprylin	56.2	39.5	4.3
Tri-caprin	27.7	63.0	9.3
Tri-laurin	26.5	70.0	3.5
Tri-myristin	9.2	87.6	3.2
Tri-palmitin	1.7	98.3	0.0
Tri-olein	74.8	19.3	5.9
Tri-stearin	0.0	100.0	0.0

Action on simple tri-glycerides. The results obtained on the simple tri-glycerides with 119 cultures are summarized in table 1. From the data it is evident that, as determined by Nile-blue sulphate, the hydrolysis of the simple tri-glycerides of the saturated fatty acids became more difficult as the molecular weight increased. Tri-propionin was hydrolyzed by 98.3 per cent of the cultures and tri-stearin by none of them. A considerably larger percentage of the organisms hydrolyzed tri-propionin than any of the other simple tri-glycerides. Tri-olein was hydrolyzed by 74.8 per cent of the cultures, a percentage that is only slightly

lower than the percentage that hydrolyzed tri-butyrin; the detailed data show that there were a few cultures which hydrolyzed one of these tri-glycerides but not the other. It is of interest to note that the tri-glycerides of the fatty acids containing an uneven number of carbon atoms were hydrolyzed by considerable percentages of the organisms; however, tri-heptylin was hydrolyzed by a smaller percentage of the organisms than tri-caproin or tri-caprylin, and tri-iso-valerin by a smaller percentage than tri-n-valerin.

TABLE 2

The hydrolysis of some natural and hydrogenated fats by 92 cultures of bacteria as indicated by Nile-blue sulphate

NATURAL FAT OR OIL	PER CENT OF CULTURES SHOWING		
	Hydrolysis	No hydrolysis	Questionable hydrolysis
Beef fat	83.7	15.2	1.1
Butter fat	80.4	18.5	1.1
Cocoanut oil	77.2	21.7	1.1
Corn oil	84.8	15.2	0.0
Cottonseed oil	78.3	20.6	1.1
Lard	86.0	14.0	0.0
Linseed oil	83.7	15.2	1.1
Olive oil	82.6	16.3	1.1
Hydrogenated cottonseed oil	79.2	20.8	0.0
Hydrogenated lard	79.2	20.8	0.0

Action on natural and hydrogenated fats. The results secured on natural and hydrogenated fats with 92 cultures are summarized in table 2. The data indicate that, as shown by Nile-blue sulphate, there was little variation in the percentage of the organisms that hydrolyzed the different materials, the values ranging from 77.2 to 86.0 per cent; cocoanut oil was hydrolyzed by the smallest percentage of the organisms and lard by the largest percentage. The detailed data for the individual organisms indicate that when an organism hydrolyzed one of the fats it also hydrolyzed most of the others. The hydrogenation of cottonseed oil and lard did not significantly influence the action of the bacteria on them.

DISCUSSION OF RESULTS

The difference between the color change produced by Nile-blue sulphate in certain fatty acids and that produced in certain simple tri-glycerides and in natural fats forms the basis of a convenient test for detecting the ability of organisms to hydrolyze fat when dispersed in an agar medium. While with natural fats the color change is the principal factor to be taken into consideration, with certain of the simple tri-glycerides the disappearance of the globules without the formation of a blue color may occur because of the solubility of the acids.

The presence of the indicator in the medium is convenient because it permits repeated observations on the action of organisms. It should be recognized, however, that Nile-blue sulphate may inhibit the growth of some organisms under certain conditions.

The variations in the action of bacteria on the simple tri-glycerides is much greater than the variations in the action on natural fats. This suggests that if differences in the lipolytic powers of bacteria are to be used in classification studies they should be investigated with simple tri-glycerides rather than mixed tri-glycerides.

SUMMARY

1. When dispersed in agar containing Nile-blue sulphate, tri-propionin, tri-butyrin, tri-caproin, tri-caprylin and tri-olein were colored bright red, and tri-caprin, tri-laurin, tri-myristin, tri-palmitin and tri-stearin were colored red to a degree which decreased rapidly with the increase in the melting points, until very little red color was present. Under similar conditions, caproic, caprylic and oleic acids were colored uniformly blue, capric and lauric acids varied in the intensity of the blue, while myristic, palmitic and stearic acids absorbed very little of the blue; the blue color was especially intense with oleic acid.

2. When dispersed in agar containing Nile-blue sulphate, beef tallow, butter fat, cocoanut oil, corn oil, cottonseed oil, lard, linseed oil and olive oil were colored bright red; linseed oil and

olive oil did not appear as red as the others, due presumably to the small size of the globules.

3. With tri-caproin and tri-caprylin, hydrolysis by bacteria was more readily detected by the disappearance of the globules than by the color change with Nile-blue sulphate. With natural fats the disappearance of the globules was not clearly evident, and the results were based on the color change.

4. As shown by Nile-blue sulphate, the hydrolysis by bacteria of various simple tri-glycerides and natural fats was not appreciably affected by (*a*) the manner in which the materials were dispersed or (*b*) the pH of the medium within the limits used; however, the more alkaline reaction favored the growth of the lipolytic bacteria studied.

5. Tri-propionin was more easily hydrolyzed by bacteria than various other simple tri-glycerides or natural and hydrogenated fats.

6. In general, the hydrolysis by bacteria of simple tri-glycerides of the saturated fatty acids was more difficult as the molecular weight increased. Tri-olein was comparatively easily hydrolyzed.

7. In general, each of the organisms used had much the same action on various natural and hydrogenated fats.

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The American Type Culture Collection

In 1925 a collection of bacteria, maintained for 11 years by the American Museum of Natural History in New York City, and after 1922 at the Army Medical Museum in Washington, D. C., was established in the John McCormick Institute for Infectious Diseases in Chicago as the American Type Culture Collection. It is administered by a committee representing the Society of American Bacteriologists, the American Phytopathological Society, the Society of Pathologists and Bacteriologists, the Society of American Zoologists, and the McCormick Institute.

The collection maintained at the McCormick Institute contains at the present time about 1300 cultures representing all branches of bacteriology. In addition, connections have been established with other laboratories at home and abroad which have made a large number and variety of bacteria, yeasts, and fungi available for distribution.

In the 9 years that the collection has been under the present management over 34,000 cultures have been distributed. The greater part of these have gone to institutions mostly for teaching purposes, but the calls for cultures for industrial purposes have increased materially. The collection has been able to be of additional service by obtaining cultures for special purposes from laboratories in this country and Europe.

The collection was established on a grant from the General Education Board, but since the expiration of this fund it has been supported by the income from the sale of cultures supplemented by a small grant from the Society of American Bacteriologists and a few contributions from industrial companies. Under these conditions it has been necessary to exercise rigid economy in the management of the collection, but this has been done without any serious impairment of its efficiency and only a small reduction in the number of available cultures.

With the removal of substantial support from outside sources, it became necessary to increase the price of cultures and this has, as would be expected, caused a falling off in the number of cultures sold. The depressed business conditions have also affected the sale of cultures adversely, but in spite of these unfavorable conditions nearly 3000 cultures were distributed in the past year, and the committee was able to meet all obligations and finish the year with a small balance.

It is the aim of the committee to make available to the investigator and teacher authentic cultures of the greatest range possible of bacteria, yeasts, and fungi. Without the aid of funds aside from those coming from the sale of cultures, further expansion of the collection is impossible. When funds are available protozoa will be added.

Since, under present conditions, it seems possible to provide this service only through the sale of cultures, it is hoped that those interested will call the attention of their friends and correspondents to the service offered by the collection, which may be addressed at 637 South Wood Street, Chicago, Illinois.

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