THE ANTISTREPTOCOCCAL PROPERTY OF MILK

II. The Effects of Anaerobiosis, Reducing Agents, Thiamine, and Other Chemicals on Lactenin Action

By ARMINE T. WILSON. M.D., and HERMAN ROSENBLUM, M.D.

(From the Alfred I. du Pont Institute of the Nemours Foundation, Wilmington)

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In order to gain, if possible, some insight into the nature of lactenin and its mode of action, studies have been made of the effects of modified physical conditions and of chemical agents on it. Certain superficial similarities exist between lactenin and Tillett's acute phase serum inhibitor of streptococcal growth (1). One of the striking properties of the latter agent is its inactivation by anaerobiosis and reducing substances and lactenin has been investigated to see whether it would be similarly affected. Studies have also been made to determine the effect, if any, of enzyme inhibitors, thiol reagents, and other chemicals. Inasmuch as some antibacterial substances appear to operate by the mechanism of competetive inhibition and are counteracted by an excess of the growth factor with which they compete, the effect of adding large amounts of vitamins and nutrilites to lactenin has been studied.

Methods and Materials

The basic methods for studying lactenin action have been described in the first paper of this series (2). Many of the experiments described here have been performed with strain 327W, a representative lactenin-sensitive organism which belongs to group A, type 1, and was originally isolated in a milk-borne epidemic. Strain 090R, a group B organism isolated from a human throat, has been used as a representative lactenin-resistant strain.

To test the effects of chemical agents on lactenin activity, series of fresh and boiled milk agar plates were prepared, containing solutions of the chemical agents in such strength as to give the desired range of final concentrations. The plates were streaked with four streptococcal strains known to be highly sensitive to lactenin and with four others known to be highly resistant; and were inspected after incubation for 24 to 48 hours. Inactivation of lactenin by the added chemical was indicated by growth of the sensitive strains on the fresh milk plate. Inhibition of bacterial growth by the chemical itself was indicated by failure of growth on both plates. As an alternative to this method, solutions of the chemical agents were added to fresh and boiled sterile liquid milk to give the desired final concentrations, the tubes were given a small inoculum of streptococcus (usually 10^{-5} ml. of blood-broth culture to 2 ml. of the mixture) incubated 16 to 20 hours at 37°C. and a loopful streaked on blood agar.

EXPERIMENTAL

Effect of Anaerobiosis on Lactenin Action.—It was discovered that lactenin, unlike Tillett's inhibitor, failed to be inactivated when a vaseline seal was

placed over the milk during incubation. However, when more rigid exclusion of oxygen was accomplished, lactenin was unable to act.

Fresh milk agar plates were inoculated with a lactenin-sensitive (327W) and a lactenin-resistant (090R) streptococcus, each strain being spread over ¼ of a plate, the remaining half being uninoculated. The plates were then placed in a jar which was rendered anaerobic by burning phosphorus. Boiled milk agar plates in the jar served as lactenin-free controls, and a set of fresh and boiled milk agar plates similarly inoculated was incubated aerobically. Uninoculated fresh and boiled milk agar plates to which methylene blue had been added were placed in the jar to serve as an indicator of the efficacy of the anaerobiosis. After incubation for 48 hours, the anaerobic jar was opened and the plates were inspected.

The methylene blue plates were found to be fully reduced. Growth of the lactenin-sensitive strain was as good on the fresh milk agar plate as on the boiled milk agar plate, indicating that under the anaerobic conditions of the jar the lactenin had been inactive. That the milk contained lactenin was shown by inhibition of growth of the lactenin-sensitive strain on the corresponding fresh milk agar plate which had been incubated aerobically. Both strains grew well on the boiled milk agar plates, whether incubated aerobically or anaerobically.

When the plates which had been in the anaerobic jar were removed from the jar they became oxidized within a few minutes as was shown by restoration of color to the plates containing methylene blue. To determine whether lactenin would be reactivated when this happened, the same plates which had been tested under anaerobic conditions were again inoculated with the test strains, each strain being spread over an unused 1/4 of the plate. The plates were incubated aerobically and were inspected after 24 and 48 hours. It was found that on reoxidation the fresh milk agar had recovered its ability to inhibit growth of the lactenin-sensitive strain. It was apparent that lactenin was reversibly inactivated by the exclusion of atmospheric oxygen.

Effect of Chemical Reducing Agents on Lactenin.—A study was then made to determine whether chemical reducing agents would effect a result similar to anaerobiosis.

Tubes of fresh and boiled milk incorporating varying quantities of reducing chemicals were inoculated with lactenin-sensitive and lactenin-resistant streptococci, incubated aerobically at 37°C. overnight and subcultured to determine whether lactenin had been active.

It was observed (Table I) that cysteine, sodium thioglycollate, glutathione, and BAL (2,3-dithiopropanol) in sufficient quantity completely abolished the inhibitory effect of lactenin on sensitive streptococci, and that even in the highest concentration used they had no antibacterial effects themselves. Sodium ascorbate appeared to have no inactivating effect but it is difficult to be certain that the ascorbate was not destroyed under the conditions of the test.

The inhibition of lactenin by anaerobiosis as well as by reducing chemicals suggests that oxidation-reduction potentials are intimately concerned with its

inhibitory power. It is possible that an E_h of a particular value and above is required for lactenin to act. We have been unable to investigate this point directly by measurement of the potentials in milk after the addition of graded amounts of a reducing agents because of the serious difficulties involved in interpreting oxidation-reduction potentials in complex biological fluids. For example, Jackson (3) demonstrated that milk reduced methylene blue visually when the E_h by electrode measurements was as high as +0.200 volts, although

TABLE I

Effect of Reducing Agents on Lactenin Action

The reducing agents were dissolved in distilled water, brought to pH 7.0, sterilized by filtration, and added to sterile fresh and boiled milk to give the desired concentrations in 2.0 ml. of mixture. The tubes were inoculated with 10^{-5} ml. of 18 hour broth culture, incubated overnight, and subcultured on sheep blood-agar. ++++ = maximal growth; − = no growth. The BAL, being relatively insoluble in water, was distributed as a stable suspension.

Reducing agent	Test strain	Growth					Growth				
		Concentration in mg. per 100 ml. of Fresh milk					Concentration in mg. per 100 ml. of Boiled milk				
		100	10	1	0.1	0.01	100	10	1	0.1	0.01
Sodium thioglycollate	327W	++++	++++	−	−	−	++++	++++	++++	++++	++++
	090R	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Cysteine HCl	327W	+++	++	−	−	−	++++	++++	++++	++++	++++
	090R	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Glutathione	327W	++++	++++	−	−	−	++++	++++	++++	++++	++++
	090R	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
BAL	327W	++++	++++	++++	++	+	++++	++++	++++	++++	++++
	090R	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Sodium ascorbate	327W	−	−	−	−	−	++++	++++	++++	++++	++++
	090R	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

the E_o of methylene blue is considered to be +0.017 volts at pH 6.8. Similarly Thornton and Hastings (4) found that complete visual reduction of methylene blue in different milk samples occurred at E_h values between +0.075 and +0.225 as determined electrometrically. From our experience we can say that lactenin has always been inactive when the E_h has been low enough to decolorize methylene blue, either by removing oxygen or by adding chemical reductants.

The inactivating effect of the sulfhydryl compounds cysteine, BAL, glutathione, and thioglycollate could be due to their ability to reduce the E_h below a critical level or it could result from their ability to combine with traces of metallic elements, if such were essential to lactenin action. If the latter were the case,

other metal-binding compounds such as sodium pyrophosphate should also abolish lactenin activity. Lactenin, however, is not injured by them.

The Bactericidal Nature of Lactenin Action.—The ability to inactivate lactenin by adding reducing agents to milk makes it possible to determine whether streptococci are dead after lactenin action or whether they have merely been prevented from multiplying.

50 ml. of fresh milk having a low lactenin titre was inoculated with 10^{-4} ml. of an 18 hour blood-broth culture of strain 327W, and colony counts showed that the milk received 1,580 organisms per ml. After standing in the incubator at 37° C. for 24 hours, colony counts were made of the milk, and it was found to contain 70 organisms per ml. Glutathione in a final concentration of 100 mg. per 100 ml. was added to the milk culture and after an additional 24 hours incubation colony counts showed that the surviving but non-multiplying streptococci had been able to proliferate after addition of the reducing agent, there being 20,200,000 organisms per ml.

Another specimen of milk, with a higher lactenin titre, was inoculated with 10^{-4} ml. of 327W culture and incubated 24 hours. Subcultures in sheep blood agar at that time failed to show growth, indicating that the streptococci had been killed. It could be argued, however, that lactenin having been transferred to the blood agar plate, continued to exert a suppressive action on the streptococci. Therefore, glutathione was added to the original milk culture to inactivate the lactenin in it, and it was then incubated 24 additional hours.

Colony counts at that time showed no growth, demonstrating that the streptococci had been killed and not merely prevented from multiplying. Lactenin may be said, therefore, to be bactericidal in its effect, if a bactericidal agent is considered one which, under the experimental conditions employed leads to death of the organism.

Effect of Other Chemical Reagents on Lactenin and on Streptococcal Growth.—The inactivation of lactenin by the exclusion of oxygen was reversed by readmitting oxygen. We desired to determine whether the inactivation produced by chemical reducing agents could also be reversed in this case by adding oxidizing agents. This could not be properly done because it was found that the oxidizing agents themselves, in a critical concentration, were toxic to lactenin-sensitive strains but not to lactenin-resistant strains, and it could not be decided whether the inhibition of growth was due to reactivated lactenin or to the agents themselves. Hydrogen peroxide, sodium iodoacetate and sodium arsenite reacted similarly in this regard (Table II). These three compounds share the property of attacking sulfhydryl groups. It was considered that lactenin also might function, possibly as an oxidizing agent, in attacking sulfhydryl groups within the streptococcal cell.

A number of other chemicals known to have biological effects were tested for their ability to inactivate lactenin and to suppress streptococcal growth. Hydroxylamine, sodium fluoride, sodium azide, sodium pyrophosphate, and sodium selenite did not inactivate lactenin but, over a narrow zone of concentration, selectively inhibited the growth of lactenin-sensitive organisms. Ure-

thane, 2,4-dinitrophenol, methionine, and carbon monoxide did not inactivate lactenin and, when they suppressed streptococcal growth, did so to lactenin-resistant as well as to lactenin-susceptible strains. The varied nature of the compounds which are able at a particular concentration to act like lactenin sug-

TABLE II

Effect of Sulfhydryl-Reactive Agents on Lactenin and on Streptococcal Growth

The chemical reagents were dissolved and diluted in distilled water and brought to pH 7.2, then added to fresh and boiled milk-agar and plates poured. Plates were inoculated on the surface with loopful of 18 hour blood-broth cultures and incubated overnight at 37° C. ++++ = maximal growth; − = no visible growth.

Agent	Test strain	Group	Growth: Concentration in mg. per 100 ml. of Fresh milk					Growth: Concentration in mg. per 100 ml. of Boiled milk				
			100	10	1	0.1	0.01	100	10	1	0.1	0.01
Sodium arsenite	327W	A	−	−	−	−	−	−	−	++++	++++	++++
	D205	A	−	−	−	−	−	−	−	++++	++++	++++
	D58/44/2	A	−	−	−	−	−	−	−	++++	++++	++++
	J17D	A	−	−	−	−	−	−	−	++++	++++	++++
	090R	B	−	+++	++++	++++	++++	−	++++	++++	++++	++++
	K106	C	++	++++	++++	++++	++++	−	++++	++++	++++	++++
	D76	D	++	++++	++++	++++	++++	−	++++	++++	++++	++++
	C133	G	−	++++	++++	++++	++++	−	++++	++++	++++	++++
Hydrogen peroxide	327W	A	−	−	−	−	−	−	−	+++	+++	+++
	D205	A	−	−	−	−	−	−	−	+++	+++	+++
	D58/44/2	A	−	−	−	−	−	−	−	++	++	±
	J17D	A	−	−	−	+	+	−	−	+++	+++	++++
	090R	B	−	++++	++++	++++	++++	−	++++	++++	++++	++++
	K106	C	−	++++	++++	++++	++++	−	++++	++++	++++	++++
	D76	D	−	++++	++++	++++	++++	−	++++	++++	++++	++++
	C133	G	−	++++	++++	++++	++++	−	++++	++++	++++	++++
Sodium iodoacetate	327W	A	−	−	−	−	−	−	−	+	+++	++++
	D205	A	−	−	−	−	−	−	−	−	++	++++
	D58/44/2	A	−	−	−	−	−	−	−	++	+++	++++
	J17D	A	−	−	−	−	−	−	−	−	+++	++++
	090R	B	−	−	−	++++	++++	−	−	+++	++++	++++
	K106	C	−	−	−	++++	++++	−	−	+++	++++	++++
	D76	D	−	−	−	++++	++++	−	−	+++	++++	++++
	C133	G	−	−	−	++++	++++	−	−	+++	++++	++++

gests that lactenin-sensitive organisms are less resistant to injury by a number of toxic agents acting through diverse mechanisms, than are the lactenin-resistant strains.

Effect of Thiamine on Lactenin Action.—Since many antibacterial agents injure susceptible cells by the mechanism of competitive inhibition and are rendered harmless by an excess of the substance with which they compete, a number of growth factors were studied for their ability to interfere with lac-

tenin action. It was not thought that lactenin itself would act as a competitor of this type, since it appears that lactenin is a large molecule and most of the known competitors have a molecular size approximately similar to that of the corresponding growth factors. Nevertheless, it was conceivable that the action of lactenin might result in the formation of a smaller molecule which could function in substrate competition.

Glutamine, riboflavin, thiamine, pyridoxine, biotin, folic acid, calcium pantothenate, nicotinic acid, guanine, xanthine, and *para*-aminobenzoic acid were added to milk-agar plates in quantities greatly exceeding those normally required for bacterial growth. The plates were then inoculated with several lactenin-susceptible and lactenin-resistant streptococci and incubated overnight.

TABLE III

The Effect of Thiamine on Lactenin Action

Tubes containing fresh milk diluted in boiled milk to the indicated degree and containing the indicated concentration of thiamine (neutralized) in 2.0 ml. of final mixture were inoculated with 10^{-5} ml. of an 18 hour blood-broth culture of the lactenin-sensitive strain 327W, and were incubated at 37° C. for 24 hours. A loopful from each tube was streaked on blood-agar and incubated overnight. ++++ = maximal growth; — = no growth. The figures in parentheses indicate the number of individual colonies which grew from the inoculum.

Dilution of fresh milk	Growth				
	Thiamine, *mg. per 100 ml.*				
	3.0	1.5	0.75	0.38	0
1:2	—	—	—	—	—
1:4	(9)	(1)	—	—	—
1:8	++++	(1)	—	—	—
1:16	++++	++++	++++	(44)	(70)
1:32	++++	++++	++++	++++	++++
Boiled milk only	++++	++++	++++	++++	++++

It was found that of the substances tested, only thiamine interfered with lactenin action. For verification of this preliminary observation, tubes of fresh milk containing graded amounts of lactenin and graded amounts of thiamine were inoculated with streptococcus 327W, incubated overnight, and a loopful from each tube was then streaked on a blood-agar plate. It was seen that in liquid milk, as on milk-agar plates, thiamine prevented lactenin action, and that in a rough way, the more lactenin present the more thiamine was required to cause the inactivation (Table III).

This observation immediately suggested the thought that lactenin is an agent which denies thiamine to the susceptible cell or that in the presence of a great excess of thiamine the streptococcus is able to make a metabolic bypass around an injurious, lactenin-induced reaction.

If the nutritional functions of thiamine are involved in this phenomenon, one difference between lactenin-susceptible and lactenin-resistant strains might be that the former require exogenous thiamine for growth whereas the latter do not. Unfortunately, adequate studies of the thiamine requirements of the various streptococci have not been made, and, indeed, are difficult to undertake because of the lack of a medium which, containing no thiamine, supplies the other factors necessary for growth from a small inoculum of all the streptococcal strains it is desired to study.

The medium of Bernheimer, Gillman, Hottle, and Pappenheimer, Jr., (5), when supplemented with xanthine and folic acid and when given a very large inoculum, will support the growth of a number of streptococcal strains, and we have succeeded in growing in this medium 14 strains, representing several serological groups and varying degrees of lactenin susceptibility. It was possible to demonstrate differences in the growth of these strains when the medium did and did not contain thiamine.

The medium, with minor modifications, was prepared according to the directions of Bernheimer, *et al.* "Vitamine-free" casein hydrolysate (General Biochemicals Corp.) served as the source of amino acids. Xanthine was added to a final concentration of 1 mg. per 100 ml. and folic acid to a concentration of 1.0 μg. per 100 ml. The medium was distributed in 5.0 ml. amounts in two series of tubes. In one series thiamine was omitted; in the other it was included in a final concentration of 0.15 μg. per 100 ml.

The organisms used for inoculation were grown overnight in 2.0 ml. of neopeptone, meat-infusion, serum broth containing no added dextrose. In the morning 5 ml. of neopeptone meat-infusion broth containing 0.2 per cent dextrose and 1 per cent normal rabbit serum was added to the overnight culture and incubation proceeded for 4 additional hours. The tubes were then centrifuged, the supernatant broth drawn off and the sedimented organisms were suspended in 7.0 ml. of sterile 0.85 per cent sodium chloride solution. One drop (approximately 0.04 ml.) of this suspension was used to inoculate the tubes of test media which were then incubated 18 hours. Growth was estimated turbidimetrically in the Coleman, Jr., photoelectric colorimeter using 10 mm. round cuvettes, light of wave length 4750 Å, and uninoculated medium as the reference standard.

All the strains grew better when thiamine was present than when it was omitted from the medium except the 3 group D strains (Table IV). Strains 327W, K2, C496 and D167C in particular were stimulated by thiamine. Only one of these (327W) is lactenin-sensitive; two of the others (C496 and D167C) are as resistant to lactenin as any strains we have tested. Strain K2 is one which, although initially inhibited by lactenin ultimately grows well (see the first paper of this series (2), Table III). Strain C998/31/3, a group A strain, is fully sensitive to lactenin, but showed no better growth response to thiamine than did the lactenin-resistant strains 090R, K106, and others. It is apparent from these results that sensitivity to lactenin is not dependent upon a need for (or the ability to have growth enhanced by) exogenous thiamine.

Thiamine is present in fresh cow milk in several forms. It exists as free thia-

TABLE IV

Effect of Thiamine on Growth of Hemolytic Streptococci in Partially Defined Medium

Strain	Group	Lactenin sensitivity	Growth in complete medium	Growth in medium without thiamine	Ratio of growth with thiamine to growth without thiamine
327W	A	S	0.078	0.007	11.25
C998/31/3	A	S	0.106	0.086	1.23
090R	B	R	0.219	0.108	2.30
K2	B	R	0.190	0.011	17.30
K4	B	R	0.170	0.156	1.09
C496	C	R	0.138	0.023	6.00
K106	C	R	0.158	0.117	1.35
D76	D	R	0.255	0.271	0.94
D178A	D	R	0.281	0.292	0.96
D178B	D	R	0.289	0.320	0.90
F90D	H	I	0.178	0.125	1.43
D34F	K	R	0.190	0.174	1.09
D167C	L	R	0.185	0.009	20.3
K130	L	I	0.128	0.104	1.23

Growth was measured turbidimetrically and is expressed as optical density (O.D. = − log transmittance). S = sensitive; R = resistant; I = intermediate sensitivity.

TABLE V

Effect of Thiamine on Inhibition of Growth of Lactenin-Sensitive Streptococcus by Hydrogen Peroxide

30 per cent H_2O_2 was diluted in distilled water and added to 2.0 ml. boiled milk containing graded amounts of thiamine to give desired final concentrations. Tubes were inoculated with 10^{-5} ml. of 18 hour blood-broth culture of lactenin-sensitive strain 327W. Loopful from each tube streaked on blood-agar plate after 24 hours' incubation at 37° C. ++++ = maximal growth; − = no growth. Figures indicate number of colonies developing from loopful streaked on plate.

H_2O_2	Growth			
	Thiamine, *mg. per 100 ml.*			
	100	10	1	0
mg. per 100 ml.				
400	−	−	−	−
200	−	−	−	−
100	1	−	8	1
50	++++	++++	++++	++++
25	++++	++++	++++	++++
0	++++	++++	++++	++++

mine, as a protein-thiamine complex, and as cocarboxylase or a protein-cocarboxylase complex. It is tempting to postulate that the lactenin in fresh milk is combined with thiamine, that such a complex cannot be utilized by lactenin-

susceptible streptococci and that heat destroys the complex, liberating thiamine in a usable form. However, this is not likely to be the case, since the amount of free, readily dialysable thiamine in fresh milk (about 23 μg. per 100 ml.) (6), is fully adequate for streptococcal growth. The function of lactenin cannot, therefore, be to hold the thiamine in milk in a combined form which renders it inaccessible to the streptococcal cell. Lactenin differs in several important respects from thiaminase, in particular its failure to be inhibited by cyanide and fluoride (7). Furthermore, after dialysis thiaminase is reactivated by glutathione, whereas lactenin is inactivated by glutathione.

The impressive fact that the selective attack of lactenin on particular streptococcal strains, as shown earlier in this paper, can be duplicated by certain simple chemical reagents in critical concentration suggested that it might be worth while to determine whether thiamine could also prevent the harmful reaction produced by those agents. With this in mind, an experiment was conducted to determine whether thiamine in high concentration would protect the susceptible streptococcal cell from hydrogen peroxide (Table V). No such protective effect was observed and it was concluded that the protective action of thiamine was specifically directed towards lactenin and not towards all reagents which selectively inhibit lactenin-sensitive streptococci.

DISCUSSION

Since lactenin has not yet been isolated in pure form, its chemical nature has not been determined. The heat lability and non-dialysability of lactenin are properties shared by many enzymes, but there is no direct evidence that lactenin is an enzyme. Hanssen (8) suggested that oxidases and peroxidases were responsible for the antibacterial action of milk, since the disappearance of the bactericidal power of milk under the influence of heat was accompanied by lost ability of the milk to react in the *para*-phenylenediamine reaction for oxidases. We do not believe lactenin to be identical with the oxidases since the latter are readily inactivated by sodium cyanide whereas lactenin is unaffected by it. Hanssen's test bacteria were *Salmonella typhosa* and *Salmonella paratyphosa A*, so that the phenomenon with which he was dealing and the antistreptococcal property of milk with which we are concerned may be due to quite different agents. Green and Pauli (9) demonstrated that xanthine oxidase from milk inhibited the growth of group A streptococci as a result of the accumulation of H_2O_2 through the action of the enzyme on its substrate. Lactenin is not identical with xanthine oxidase because unlike that enzyme it is active in the presence of high concentrations of beef liver catalase, is not inhibited by sodium cyanide, and is present in apparently identical form in human milk, which is known not to contain xanthine oxidase. Lysozyme, which is present in small amounts in cow milk, is not identical with lactenin because preparations of milk which contain too little lysozyme to cause lysis of *Micrococcus lysodeikticus* nevertheless have excellent lactenin action, and because lysozyme

in high concentration, as in egg white, fails to inhibit the growth of group A streptococci. If lactenin is an enzyme it must, therefore, be distinct from the known antibacterial enzymes of milk.

The fact that lactenin is growth-inhibitory only in the presence of molecular oxygen suggests that its action may be to promote a harmful oxidation in the susceptible cell, and that those cells which are not inhibited by lactenin either fail to be injured by the oxidation or cannot enter into the postulated reaction. The lactenin-inactivating effect of the sulfur-containing reducing agents appears to reside in their ability to reduce the oxidation-reduction potential, thus preventing the postulated oxidative injury, rather than in their ability to combine with metallic elements.

It is possible that lactenin exists in oxidized and reduced forms, and that it is active only in the oxidized state. If such is the case, the inactivation of lactenin by the exclusion of atmospheric oxygen would result from its being held in the reduced form by the low E_h which develops when milk is denied contact with gaseous oxygen. Similarly the low potential produced by the addition of chemical reductants would convert lactenin to a reduced, inactive form.

The influence of oxidation-reduction potentials on lactenin has important bearing on the functioning of lactenin *in vivo*, which will be taken up in the third paper of this series.

The ability of thiamine to counteract lactenin is of considerable interest although the significance of the observation is not understood. Thiamine alone of the growth factors tested had such an action. That, together with the fact that thiamine failed to protect against injury by hydrogen peroxide suggests that its relation to lactenin may be specific.

Thiamine is known to inhibit the action of diamine oxidase. Lactenin does not appear to be a diamine oxidase, however, because choline, which also inhibits that enzyme, is without effect on lactenin, and because there is no diamine oxidase in cow milk (10).

If the action of lactenin is to deny thiamine to the susceptible cell, it could do so by several mechanisms. It could, for example, act as a thiamine-binding substance or as a thiaminase. The fact that free thiamine exists in lactenin-containing milk is opposed to these possibilities, yet it must be remembered that thiamine also exists in tissues containing thiaminase, the enzyme being inhibited in some manner from destructive action (7). It is possible that lactenin may penetrate the susceptible cell and that inside the cell, separated from inhibitory influences of other substances in milk, it could destroy thiamine; or that intracellularly it could unit with thiamine, forming a complex which the cell could not utilize. These possibilities must be considered highly speculative until more work is done. The concept that lactenin operates within the cell is consistent with the observation that requirement for exogenous thiamine is not related to lactenin sensitivity.

We have performed some preliminary experiments with a limited amount of pyrithiamine (neopyrithiamine, Merck and Co., Inc.). The object of these experiments was to determine whether pyrithiamine, a thiamine antagonist, and lactenin, possibly a thiamine antagonist, would inhibit the same strains of streptococcus. All the streptococcal strains tested were sensitive to pyrithiamine in high concentration, but some were much more sensitive than others. In general the strains which were shown by growth studies in the partially defined medium to be most stimulated by thiamine were most readily inhibited by pyrithiamine. There was, however, no consistent direct correlation between a requirement for thiamine and pyrithiamine sensitivity on the one hand and sensitivity to lactenin on the other.

The amount of thiamine required to inactivate lactenin is large (3 mg. per 100 ml. of milk). This is 130 times the average concentration of free thiamine in cow milk and 30,000 times the concentration of thiamine (0.01 μg. per 10 ml.) found by Pappenheimer and Hottle (11) to be optimal for the growth of streptococcus C203S in a semisynthetic medium. These quantitative relationships suggest that the mechanism of lactenin action and of its reversal by thiamine do not necessarily involve the normal nutritional function of thiamine, but that the latter may act through chemical properties of an entirely different nature.

We have made several attempts to purify lactenin, but have not succeeded in obtaining a preparation purer or more concentrated than the one of Jones and Simms (12), which on a dry weight basis was 200 to 500 times as active as whole milk; nor have we been able to develop methods superior to digestion followed by dialysis and alcoholic precipitation, which they advocated. The material obtained by these procedures is far from pure chemically. The best preparation we have obtained was made by digesting whey with crystalline trypsin and chymotrypsin, dialyzing and precipitating with 3 volumes of alcohol. This preparation contained 0.011 gm. N (0.07 gm. protein) per 100 ml. and was active against group A streptococci when diluted 1:16. If it is assumed that all the protein is lactenin, an assumption by no means valid, lactenin is active in a concentration of 4.5 mg. per 100 ml.

SUMMARY

Lactenin is reversibly inactivated by the exclusion of atmospheric oxygen. It is also inactivated by the sulfur-containing reducing agents cysteine, glutathione, thioglycollic acid, and BAL.

Group A streptococci which have been acted upon by lactenin have been killed, and not merely prevented from multiplying, since they cannot be revived by inactivating lactenin through the addition of a reducing agent.

Thiamine in great excess inactivates lactenin. The mechanism by which it accomplishes this has not been discovered, but it suggests that the mode of action of lactenin may be to deny thiamine to the lactenin-sensitive cell. Lac-

tenin sensitivity is not, however, related to a requirement for exogenous thiamine, nor does lactenin appear to function by binding environmental thiamine in a form unavailable to the sensitive cell.

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