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The Effect of some Antimetabolites (5-fluornicotinamide, Protamine, Guanazolo) on Acid Production in Vitro by Oral Micro-organisms

By

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A method suggested for the control of dental caries is the inhibition of growth or acid production of bacteria involved in the acidogenic process. A great number of substances have been tested for this purpose, *e.g.* antiseptics, antibiotics and antienzymes (APPLETON, 1950). One of the foremost in this field of research is Fosdick, who tested 381 substances for their effect on acid production (FOSDICK *et al.* 1953).

It has been observed that the majority of the lactobacillus strains associated with dental caries are homofermentative and that most of them are of the type *L. casei*. (GRUBB & KRASSE, 1953, ROGOSA *et al.* 1953). Lactobacillus strains, and especially *L. casei,* have been used extensively for investigating the metabolism of the vitamins of the B-complex. (WILLIAMS *et al.* 1950, SNELL, 1948, LANDY & DICKEN, 1942, and others). They have also been employed in the study of the synthesis of nucleic acid (HITCHINGS *et al.,* 1949, WRIGHT, 1951, ÅGREN, 1954 a). From the fields of investigation referred to above, a large number of substances, some of which are of antivitamin character,

which inhibit the growth and acid production of lactobacilli, are known.

Vitamins of the B-complex required by most lactic acid bacteria are nicotinic acid, pantothenic acid and biotine (HILL & KNIESNER, 1942, COOLIDGE *et al.* 1949, KOSER & FISHER, 1950, SNELL, 1950). Removal or lack of any of these vitamins in an otherwise complete medium causes a marked reduction in acid production by oral acidogens (DREIZEN & SPIES, 1953). An antagonist of any of these vitamins ought therefore logically to inhibit acid formation. DREIZEN & SPIES also showed that an addition of a metabolic analogue of nicotinic acid (pyridine–3-sulphonic acid) reduces the acid production by oral organisms. According to WILLIAMS *et al.*, (1950), however, *5-fluornicotinamide* is one of the most effective nicotinic acid antagonists known.

It has been shown by MILLER *et al.*, (1942) that *protamine* exerts a powerful inhibitory action on the respiration or anaerobic metabolism of various gram-negative rods, staphylococci and *B. subtilis*. It has recently been reported that protamine completely inhibits the growth of *L. casei* and *L. delbrückii* in a concentration of 0.1–1 mg per 5 ml. medium (ÅGREN, 1954 b). Protamine is a strong base and its activity against bacterial growth is supposed to be due to its reaction with nucleic acid (WRIGHT, 1951).

Guanine, a purine base, is customarily added to synthetic media and serves as a precursor for the synthesis of bacterial nucleic acids (TITTSLER *et al.*, 1952). The analogue of guanine in the triazolopyrimidine series, *5-amino-7 hydroxy-1-v-triazolo (d)-pyrimidine*, abbreviated by KIDDER *et al.*, (1949) to *guanazolo*, has been found to inhibit the growth of *E. coli* and *Staph. aureus* (ROBLIN *et al.*, 1945).

These observations called for an investigation of the effect of some antimetabolites on acid formation by oral bacteria.

Material and Methods

General

The following substances were studied: 5-fluornicotinamide,[1] protamine sulphate,[1] 5-amino-7-hydroxy-1-v-triazolo-(d)-pyrimidine (guanazolo).[1]

The effect of the various substances on bacterial metabolism was studied with the use of pure cultures and of saliva in 48 hour and 4 hour experiments. The following bacterial strains were used: *L. casei* isolated from the mouth, *L. arabinosus*, and *L. brevis*, A. T. C. C., *Str. salivarius* from State Serum Institute, Copenhagen, *Str. mitis* from the mouth, *Str. mitis* and *Str. faecalis*, A. T. C. C., *Staph. albus*, *Candida* and *E. coli* from the mouth.

Medium. — The semi-synthetic substrate described by LANDY & DICKEN (1942) was used throughout. The glucose concentration was 2 per cent. The substrate was autoclaved for 20 minutes.

Incubation. — Aerobic at 37° C. In the 48-hour tests use was made of a thermostat, in the 4-hour tests of a water-bath. The purity of the culture was checked by streaking blood agar plates.

Reading. — Acid production was determined by electrometric titration to the original pH.

Procedures

48-hour tests. — The various components were added to the substrate before the latter was autoclaved. Every test tube contained 10 ml. medium. In experiments using pure cultures, the inoculum consisted of 0.05 ml. of an 18-hour broth culture washed twice and afterwards made up to its original volume. In experiments using mixed cultures the inoculum consisted of 0.1 ml. paraffin-stimulated saliva.

4-hour tests using bacteria. — 1 ml. of test solution and 1 ml. of bacterial suspension were added to 9 ml. substrate. In the control tubes distilled water was used instead of test solution.

[1] 5-fluornicotinamide was supplied by Lilly Research Laboratories, Indianapolis, protamine sulphate by Apoteksvarucentralen, Stockholm, guanazolo by Chemotherapy Division, Stamford Research Laboratories, Stamford, Connecticut. I wish to express my gratitude for this generosity.

The bacterial suspension was obtained by centrifugation and washing of an 18-hour broth culture.

4-hour tests with saliva. — In these tests the composition of the samples was: 4 ml. paraffin-stimulated saliva, 0.5 ml. of a 40 per cent glucose solution, 0.5 ml. test solution. In the control tubes distilled water was used instead of test solution.

Results

A. *Tests with Pure Cultures*

1. *48-hour tests.* — The effect of the substances on acid formation by pure cultures is apparent from Tables 1, 2, 3 and 4.

Table 1. *The effect of 5-fluornicotinamide on acid production by different micro-organisms*
(Medium containing 0.05 γ nicotinic acid per ml. Incubation 48 hours)

Organism	Ml of acid produced		
	Control	5-fluornicotinamide mg/10 ml	
		0.1	1.0
L. casei	7.98	5.76	0
L. arabinosus	9.91	4.29	0
Str. salivarius	4.38	2.99	1.41
Str. mitis (from mouth)	1.88	0.38	0
Staph. albus	2.32	0.84	0.43
Candida	1.45	1.36	1.41

Table 2. *The effect of 5-fluornicotinamide on acid production at various concentrations of nicotinic acid*
(Strain: L. casei. Incubation 48 hours)

Nicotinic acid γ ml	Ml of acid produced		Inhibition %
	Control	5-fluornicotinamide 1 mg/10 ml	
0.26[1]	11.65	7.10	39
0.05	7.50	0.10	99
0.01	4.32	0	100

[1] 0.26 γ nic.acid/ml is the concentration of the vitamin in the medium of Landy & Dicken.

When testing the nicotinoc acid antagonist, 5-fluornicotinamide, the amount of nicotinic acid used in the medium was the smallest necessary to permit good acid formation. This concentration was found to be about 0.05 gamma nicotinic acid per ml. of the medium for most of the strains tested. This value agrees well with that found by KOSER *et al.* (1951). Table 1 shows that 5-fluornicotinamide in a concentration of 1 mg. per 10 ml. of medium effectively inhibited acid formation by *L. casei, L. arabinosus* and *Str. mitis. Staph. albus* is inhibited considerably, *Str. salivarius* much less and *Candida* not at all. It is clear from Table 2 that an increase in the quantity of nicotinic acid in the substrate to 0.26 gamma per ml. (the amount of nicotinic acid in the substrate described by LANDY & DICKEN) markedly decreased the inhibitory effect of 5-fluornicotinamide on *L. casei.*

Table 3. *The effect of protamine on acid production by different micro-organisms*
(Incubation 48 hours)

Organism	Ml of acid produced[1]			
	Control	Protamine mg/10 ml		
		0.01	0.1	1
L. casei	10.83	11.57	0	0
L. arabinosus	12.83	12.36	0	0
L. brevis	6.42	5.85	0	0
Str. salivarius	4.93	4.88	0.32	0
Str. mitis (fr. mouth)	1.84	1.95	0	0
Str. mitis (A. T. C. C.)	2.24	2.59	1.53	0
Str. faecalis	3.38	2.91	2.14	3.06
Staph. albus	3.36	3.28	2.14	0
Candida	0.94	1.00	0.94	1.18
E. coli	2.45	2.49	2.50	1.97

[1] The values are the means from two tubes.

Table 3 shows that the acid formation by the lactobacillus strains was completely inhibited by a concentration of 0.1 mg. protamine sulphate per 10 ml. substrate (0.001 per cent). All of the other strains examined with the exception of *Str. faecalis, Candida* and *E. coli,* were inhibited completely by protamine in a concentration of 1 mg. per 10 ml. medium (0.01 per cent).

A concentration of 0.01 mg. per 10 ml. produced no demonstrable effect on any of the strains tested.

In this connection it might be mentioned that the inhibitory effect of protamine was decreased in the presence of indicator in the medium (brom cresol purple) in a concentration of 0.0025 per cent. This observation agrees with that reported by MASSART & VAN DEN DAELE, (1948), who studied the effect of protamine on the respiration of yeast cells. They found a competition between protamine and basic dyes.

Table 4. *The effect of guanazolo on acid production by different micro-organisms*
(Incubation 48 hours)

Organism	Ml of acid produced		
	Control	Guanazolo mg 10 ml	
		0.1	1
L. casei	13.33	3.32	0
L. arabinosus	12.06	1.97	0.28
L. brevis	4.52	1.00	0
Str. salivarius	5.67	2.76	0.48
Str. mitis (from mouth)	0.55	0	0
Str. mitis (A. T. C. C.)	1.07	0	0
Str. faecalis	3.47	2.28	2.25
Staph. albus	2.31	2.30	1.96
Candida	0.92	0.96	0.92
E. coli	2.63	1.67	1.56

Table 4 shows that guanazolo effectively inhibited acid formation by all of the lactobacilli and streptococcal strains tested with the exception of *Str. faecalis*. The coli strain was inhibited to a certain degree but *Staph. albus* and *Candida* were not inhibited.

2. *4-hour tests.* — Some experiments were carried out to study the influence of variation in the size of the inoculum on the inhibitory effect of the various substances in 4-hour tests (Tables 5, 6 and 7). These experiments showed

a) that inhibition was greatest when the inoculum used was about 1 million bacteria per cubic millimeter,

b) that the lactobacillus strain used was inhibited much more by protamine and guanazolo than by 5-fluornicotinamide.

Table 5. *The influence of the size of the inoculum on the inhibitory effect of 5-fluornicotinamide*
(Strain: L. arabinosus. Medium containing 0.05 γ nic.acid per ml. Incubation 4 hours)

Inoculum 10^6 bact per mm^3	Ml of acid produced[1]		Inhibition %
	Control	5-fluornicotinamide 1 mg/10 ml	
0.9	1.88	1.30	31
1.8	2.47	1.99	19
3.6	3.43	2.91	15
7.2	4.97	4.49	10
14.4	7.12	6.22	13

[1] The values are the means from two tubes.

Table 6. *The influence of the size of the inoculum on the inhibitory effect of protamine.*
(Strain: L. arabinosus. Incubation 4 hours)

Inoculum 10^6 bact per mm^3	Ml acid produced[1]		Inhibition %
	Control	Protamine 1 mg/10 ml	
0.84	1.69	0.30	82
1.7	2.64	0.48	82
3.4	3.44	0.83	76
6.8	4.71	3.06	35
13.6	6.75	5.95	12

[1] The values are the means from two tubes.

Table 7. *The influence of the size of the inoculum on the inhibitory effect of guanazolo*
(Strain: L. arabinosus. Incubation 4 hours)

Inoculum 10^6 bact/per mm^3	Ml of acid produced		Inhibition %
	Control	Guanazolo 1 mg/10 ml	
0.52	1.21	0.33	73
1.04	1.35	0.41	70
2.08	4.31	1.23	71
4.16	4.56	2.21	52
8.4	6.99	2.93	58

Table 8. *The effect of protamine and guanazolo on acid production by different micro-organisms*
(Inoculum: about 1×10^6 bact/mm^3. Incubation 4 hours)

Inhibition % (at 1 mg protamine per 10 ml)	Ml of acid produced — Protamine mg/10 ml 1	Protamine mg/10 ml 0.1	Control	Organism	Ml of acid produced — Control	Guanazolo mg/10 ml 0.1	Guanazolo mg/10 ml 1	Inhibition % (at 1 mg guanazolo per 10 ml)
86	0.46	3.51	3.31	L. casei	1.41	1.22	0.99	30
85	0.42	1.37	2.89	L. arabinosus	2.28	0.96	0.84	63
34	1.76	2.43	2.66	Str. salivarius	2.72	2.45	0.98	64
67	0.48	1.49	1.47	Str. mitis (from mouth)	0.81	0.63	0	100
45	1.15	1.96	2.10	Str. faecalis	1.79	1.79	1.77	1

Some short-time experiments using various strains and an inoculum of about 1 million bacteria per cubic millimeter are summarised in Table 8. It is apparent from the table that 1 mg. protamine per 10 ml. medium produced pronounced inhibition of acid formation by the lactobacillus strains. The effect on streptococcal strains was less marked. Guanazolo also exerted an ihibitory effect, though not so strong as that of protamine.

B. *Tests with Mixed Cultures*

1. *48-hour tests.* — The effect of the various substances on acid formation in experiments using saliva inoculum is given in Table 9. The table shows that a reduction of the acid formation can be observed with the use of 1 mg. 5-fluornicotinamide in the substrate. 10 mg. guanazolo produced marked inhibition, while protamine sulphate produced only slight inhibition, even when used in such a relatively high concentration as 10 mg. per 10 ml. substrate. The experiments were repeated on some 10 other salivary samples and the results obtained were roughly the same as those given in Table 9.

Table 9. *The effect of 5-fluornicotinamide, protamine and guanazolo on acid production by mixed cultures*
(0.1 ml saliva to 10 ml medium. Incubation 48 hours)

Saliva from patient	Antimetabolite	Ml of acid produced			
		Control	antimetabolite mg per 10 ml		
			0.1	1	10
B	5-fluornicotinamide	4.33[1]		1.88	
S	»	3.97[1]		0.68	
B	Protamine	6.27	3.95	3.56	2.86
S	»	5.46	4.56	3.98	3.06
K	»	4.98	4.63	4.21	3.83
B	Guanazolo	6.55	2.90	1.99	0.20
S	»	5.60	2.76	1.27	0.47
K	»	5.60	1.90	2.35	0.74

[1] Medium used contained 0.01 gamma nicotinic acid per ml.

2. *4-hour tests.* — The formation of acid in a mixture of saliva and glucose in the presence of the substances is clear from table 10, which shows that protamine in a concentration of

Table 10. *The effect of 5-fluornicotinamide, protamine and guanazolo on acid production in a saliva-glucose mixture*

Saliva	Antimetabolite	Ml of acid produced			
		Control	Antimetabolite mg per 10 ml		
			0.1	1	10
Pooled saliva	5-fluornicotinamide	0.63	0.48	0.36	
»	»	0.57		0.34	0.33
B	Protamine	0.43	0.38	0.14	0
S	»	0.28	0.25	0.06	0
K	»	0.76	0.67	0.24	0
B	Guanazolo	0.51	0.17	0.12	0.11
S	»	0.19	0.08	0	0
K	»	0.68	0.29	0.25	0.18

0.1 per cent (10 mg./10 ml.) completely inhibited the formation of acid. Other substances also reduced acid formation, though not so much as protamine.

Discussion

The results show that the nicotinic acid analogue, 5-fluornicotinamide, the guanine analogue, guanazolo or protamine, an antimetabolite of nucleic acid metabolism can inhibit acid formation *in vitro* of pure cultures of such lactobacillus and streptococcal strains as occur in the oral flora (Tables 1–8). The acid production by mixed flora in saliva can also be inhibited (Tables 9 and 10).

It should be pointed out that the inhibitory effect of protamine is less marked in 48-hour tests with salivary inoculum than in 4-hour tests. These results appear contradictory. The poor inhibition in the 48-hour tests can, however, be explained by the assumption that some of the acid forming microorganisms in the mouth are not inhibited by protamine. This selective effect is not brought out in the short-time tests. In these tests the major portion of the acid is probably produced by streptococci, which represent the greater part of the bacteria in saliva. It is clear from Tables 3 and 8 that streptococci are inhibited by protamine.

That the substances tested could inhibit acid formation by

oral bacteria *in vitro* does not necessarily mean that they posses any caries-inhibiting effect. For a substance to be active in the mouth, it is necessary as stated by FOSDICK, (1953), for the substance to possess not only an anti-acid forming effect but also other properties: it should attach itself to the plaque and should remain active for an appreciable period of time, it should be relatively non-toxic and it should be colourless. The results obtained suggest that further investigation is desirable.

Summary

In an investigation carried out *in vitro* on oral acidogenic organisms it was observed in 4 hour and in 48 hour tests that

5-fluornicotinamide, a nicotinic acid analogue,

protamine, an antimetabolite of nucleic acid metabolism and

guanazolo, a guanine analogue

inhibited the acid formation of pure cultures of lactobacilli and streptococci as well as of mixed flora from saliva.

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