THE EFFECTS OF THREONINE ON POPULATION CHANGES AND VIRULENCE OF SALMONELLA TYPHIMURIUM

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Received for publication July 2, 1951

There is considerable evidence that nutrition is capable of modifying infections in experimental animals (Schneider, 1949; Clark, 1950), yet very little is known about the causative mechanisms of these effects. In addition, recent investigations with *Brucella* (Goodlow, Mika, and Braun, 1950) demonstrated that specific amino acids have a significant effect on bacterial population changes *in vitro* involving the establishment of variants with different virulence. It was, therefore, of considerable interest to analyze a phenomenon observed in studies (Holtman and Page, 1950) with *Salmonella typhimurium*, namely that the administration of threonine to infected guinea pigs resulted in a significant reduction of survival time. Threonine was used because it had been found by qualitative chromatographic analysis that following *Salmonella* infection the tissues of guinea pigs contained free threonine not detected in similar tissues of noninfected animals. The reasons for the virulence-enhancing effect of threonine remained obscure until it was discovered that *Salmonella* populations recovered from threonine-treated animals contained a high proportion of threonine-resistant, more virulent variants. In contrast, isolates from animals not injected with threonine yielded populations consisting primarily of less virulent cells with relative susceptibility to threonine, similar to the stock-culture population. Details of these studies and of extended investigations on the properties of the two variants with different threonine resistance will be reported here.

MATERIALS AND METHODS

The culture of *S. typhimurium* used in these experiments originated from a stock culture which has been maintained in our laboratory for several years. Information concerning the original source of this stock culture is unavailable.

Stock cultures were originally maintained on tryptose agar and later on 2 per cent glycerine, 1 per cent glucose nutrient agar. For growth in liquid media a basic synthetic medium consisting of 0.1 per cent DL-aspartic acid, 0.5 per cent NaCl, 1.72 per cent K_2HPO_4 was used, supplemented for specific experiments as will be mentioned. For observation of colony types, aliquots of liquid cultures, saline suspensions made from growth on solid media, or blood samples were streaked on McConkey agar and checked with the help of a dissecting microscope and oblique lighting (Henry, 1933) after incubation at 37 C for 48 hours. All inocula for liquid media or animals were adjusted turbidimetrically and checked by plate counts. Guinea pigs used in the virulence titrations originated from an inbred stock unselected for disease resistance or susceptibility and, unless indicated otherwise, ranged in weight from 500 to 600 g at time of infection.

RESULTS

The effect of threonine on *S. typhimurium* infection in guinea pigs was observed first in unpublished studies by Holtman and Page. Several subsequent experiments confirmed these earlier observations, and the results of one typical experiment are presented here as an example. Two groups of guinea pigs were infected intraperitoneally with *S. typhimurium*, each animal receiving 4.1×10^5 cells from a 24-hour old slant; animals of one of these groups were injected simultaneously, but at a different site, with 50 mg of DL-threonine. A third group of animals was injected with threonine only. The accumulative totals of deaths recorded daily for 15 days are presented in table 1. These data show that in the group that received the combination of amino acid and organisms a significantly greater number of animals died during the first 10 days after inoculation than in the control groups.

In order to detect whether the virulence-enhancing effect of threonine injection might be associated with population changes *in vivo*, blood samples from

TABLE 1

Survival of guinea pigs following injection of Salmonella typhimurium and threonine (average weight of animals 550 g)

GUINEA PIGS	MG OF THREONINE INJECTED	ORGANISMS INJECTED	NO. OF DAYS FOLLOWING INOCULATIONS									DEAD/TOTAL ANIMALS AFTER 15 DAYS
			1	2	3	4	5	6	7	8	9	
			Accumulative dead									
10	50	—	0	0	0	0	0	0	0	0	0	0/10
10	—	4.1×10^5	0	0	1	1	1	1	4	5	5	6/10
10	50	4.1×10^5	0	1	1	1	3	6	8	9	10	10/10

animals that died in the foregoing experiment were plated on McConkey agar and the resulting colonies were inspected. Two significantly different colonial types were noted. One was transparent, the other opaque (figure 1). *S. typhimurium* populations isolated from animals that had died after receiving a combination of threonine and organisms contained a considerable proportion of the transparent type in addition to the opaque type; isolates from animals that had received organisms only contained predominantly opaque types and only a few transparent types. Cells from either colony type failed to clump when mixed with 1:1,000 acriflavine, and the types, therefore, have been designated as smooth transparent (S^t) and smooth opaque (S^o). Picking of S^t colonies from plates streaked with blood samples resulted in the isolation of pure S^t cultures, which could be maintained without change even after repeated transfers on solid media. Similarly, pure S^o cultures were isolated; however, maintenance of pure S^o cultures required occasional reisolation of S^o colonies, since S^t types had a tendency to establish themselves in stock cultures.

Subsequent inspection of the original stock cultures of *S. typhimurium* revealed that this culture consisted primarily of S^o type cells but contained a few S^t type cells.

The *in vitro* effect of threonine on both S^o and S^t types was then investigated. To a simple synthetic L-aspartic acid medium inoculated with either S^o or S^t suspensions were added 200 μg DL-threonine per ml. Viable cell counts were made and McConkey agar plates were streaked for the detection of colonial types at regular intervals during the period of observation. The results of these experiments are summarized in table 2. The data indicate that during growth in liquid

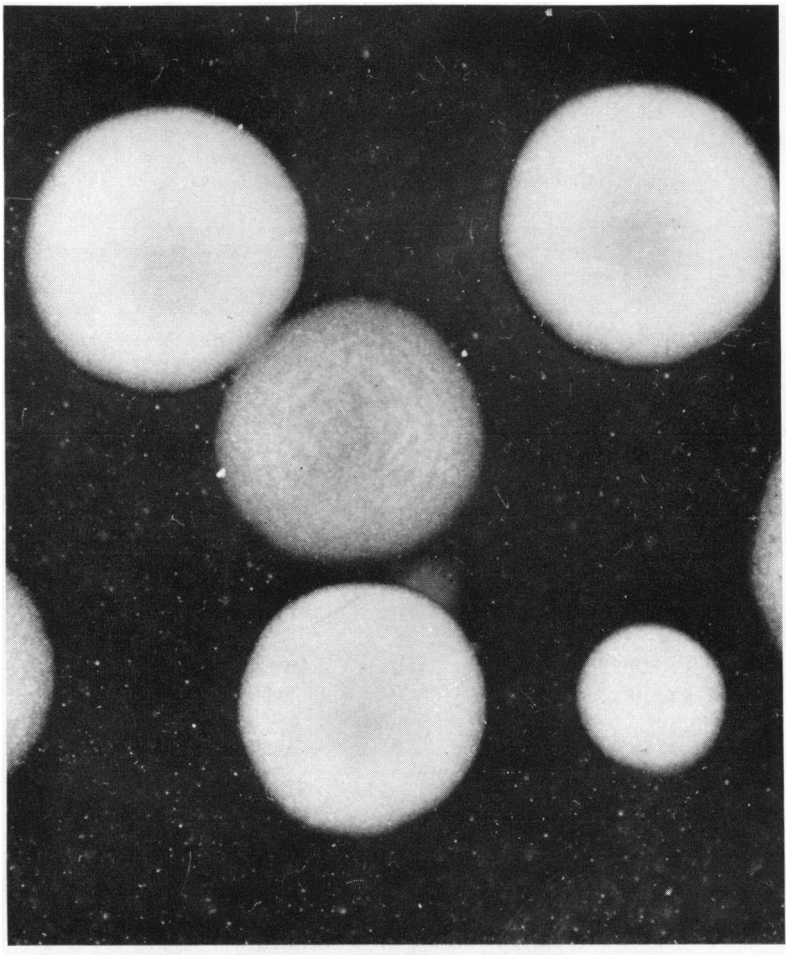

Figure 1. Four S^o colonies of *Salmonella typhimurium* surrounding an S^t colony

media the S^o type of *S. typhimurium* is relatively unstable and exhibits population changes involving the establishment of the S^t type. The addition of threonine to the medium enhances the establishment of the S^t variant. Under similar conditions, the S^t type is relatively stable and the population remains unchanged even when threonine is added to the medium. It will be noted that in contrast to the S^o type the S^t variant is not inhibited in the presence of high threonine concentration, but its growth is actually enhanced. In order to obtain additional

data on the effect of threonine upon S^o and S^t in the absence of major population changes during early growth periods, additional viable counts were made between 0 and 48 hours on DL-aspartic acid cultures containing various amounts of DL-threonine. The results are presented graphically in figures 2 and 3. They confirm that the increase in viable cells of S^o is inhibited proportionally to the amount of threonine added, as little as 50 μg per ml producing an effect. In contrast there is no inhibition of S^t in cultures containing less than 500 μg per ml of threonine; concentrations of threonine up to 200 μg per ml actually increased the number of viable cells of S^t over that observed in control cultures without added threonine.

TABLE 2

Growth of S^o and S^t Salmonella typhimurium in synthetic medium with and without addition of* DL-*threonine*

AGE, DAYS	S^o (CONTROL)			S^o + 200 μG THREONINE/ML		
	Cell count	% S^o	% S^t	Cell count	% S^o	% S^t
0	7.6×10^5	100	0	7.6×10^5	100	0
1	—	98	2	—	97	3
2	1.5×10^7	80	20	1.0×10^7	64	36
4	2.5×10^7	26	73	8.0×10^7	7	93
5	—	20	80	—	6	94
6	8.8×10^7	12	88	6.8×10^7	4	96
	S^t (CONTROL)			S^t + 200 μG THREONINE/ML		
	Cell count	% S^t	% S^o	Cell count	% S^t	% S^o
0	7.6×10^5	100	0	7.6×10^5	100	0
1	—	100	0	—	100	0
2	1.6×10^7	100	0	8.6×10^7	100	0
4	3.0×10^7	100	0	6.4×10^7	100	0
5	—	100	0	—	100	0
6	4.0×10^7	100	0	4.8×10^7	100	0

* *7B9* 0.1 per cent L-aspartic acid,
0.5 per cent NaCl,
1.72 per cent K_2HPO_4 to 1,000 ml water.

Preliminary data indicate that population changes from S^o to the threonine-resistant S^t involve the establishment of S^t mutants which arise in S^o populations at the rate of approximately 4×10^{-6} per bacterium per division cycle.

Since, as mentioned before, it had been observed that the threonine-resistant S^t type was present in large numbers in isolates from threonine-treated guinea pigs, and since these animals died earlier than the controls, it had to be determined whether the enhanced virulence effects could be attributed to increased virulence of the threonine-resistant S^t type which established itself in threonine-treated animals. The results of virulence titrations with "pure" S^t and "pure" S^o cultures are shown in table 3 and lines 2 and 4 of table 5. The data, especially

those compiled in table 3, indicate that the S^t type possesses a greater virulence for the guinea pig than the S^o type. A greater number of deaths occurred during the first four days after injection of the S^t culture. Eventually, however, the accumulative death ratios for either type of infection became approximately the same. It will be noted that in addition to the differences in virulence between S^t and S^o, the relative virulence of comparable doses of each type differed between the experiment listed in table 3 and that listed in table 5. This differ-

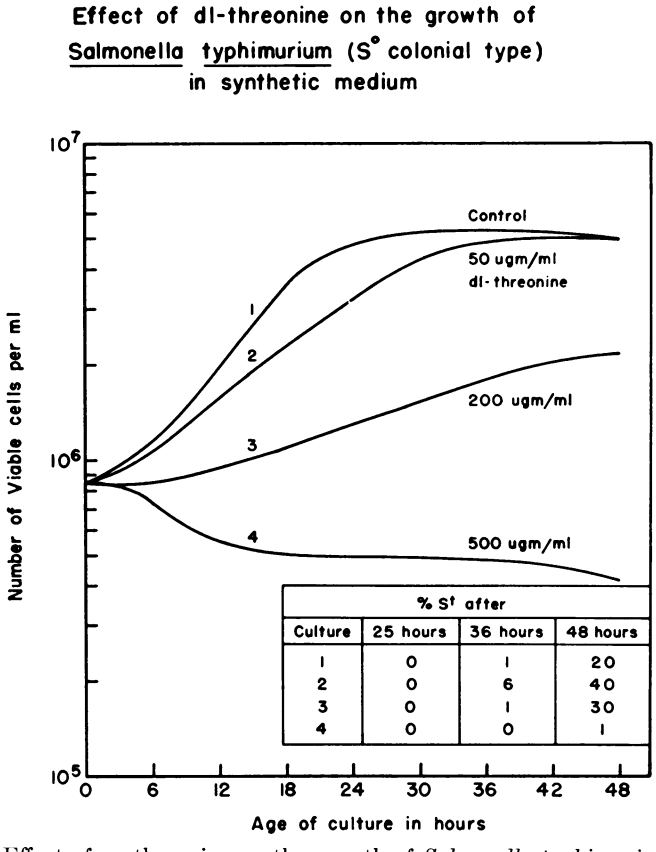

Figure 2. Effect of DL-threonine on the growth of *Salmonella typhimurium* (S^o colonial type) in synthetic medium.

ence is undoubtedly due to the difference in weight between animals used in the first experiment (average weight 550 g) and those used in the second experiment (average weight 250 g).

A series of tests was then performed to determine possible differences in the biochemical activity of both types. The results of these tests appear in table 4. The sole demonstrable difference was that S^o strain fermented raffinose after 2 days with the production of acid and gas, while the S^t type lacked this biochemical activity.

Antigenically, both strains are identical, each possessing the formula IV XII; i-1, 2, 3. . . .[1] No differences could be ascertained by agglutinin absorption tests.

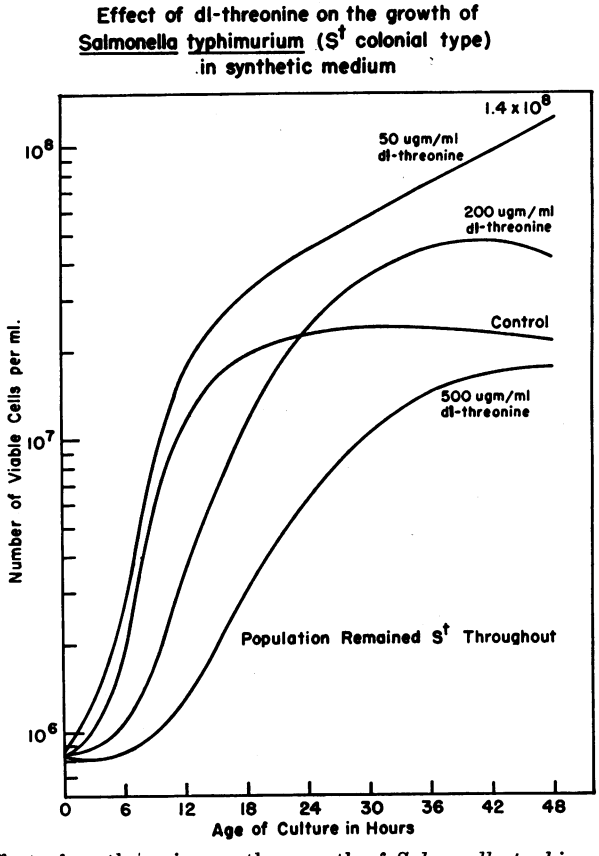

Figure 3. Effect of DL-threonine on the growth of *Salmonella typhimurium* (*S^t* colonial type) in synthetic medium.

TABLE 3

Virulence of S^o and S^t types of Salmonella typhimurium for guinea pigs (average weight of animals 250 g)

COLONIAL TYPE	ORGANISMS INJECTED	ANIMALS INJECTED	ACCUMULATIVE DEATHS BY DAYS			
			1	2	3	4
S^t	2.3×10^5	20	6	9	10	11
S^o	2.4×10^5	20	1	3	5	9

With this information on hand, the original experiments on the effects of DL-threonine injection upon *S. typhimurium* infection in guinea pigs were repeated,

[1] The authors gratefully acknowledge the cooperation of Commander L. A. Barnes, National Naval Medical Center, Bethesda, Md., in determining the antigenic formulae.

except that now pure cultures of S^t and of S^o, as well as known mixtures of S^t + S^o, were used as inoculum. Three respective groups each containing 40 animals were infected with the three inocula, and 20 animals of each group also received a simultaneous injection of DL-threonine, 50 mg per animal. The resulting survival times are presented in table 5. The data show that the virulence-enhancing effect

TABLE 4

Physiological characteristics of S^o and S^t colonial types of Salmonella typhimurium

BIOCHEMICAL TEST	S^o	S^t
L-Arabinose	AG(5)*	AG(3)
Dulcitol	—	—
Glucose	AG(1)	AG(1)
Inulin	—	—
Lactose	—	—
D-Levulose	AG(1)	AG(1)
Mannose	AG(1)	AG(1)
Mannitol	AG(1)	AG(1)
Maltose	AG(1)	AG(1)
Raffinose	AG(2)	—
L-Rhamnose	AG(1)	AG(1)
Salicin	—	—
D-Sorbitol	AG(1)	AG(1)
Sucrose	AG(2)	AG(2)
Xylose	AG(1)	AG(1)
H_2S production	++++	++++

* Figures in parentheses indicate the day on which the production of acid or gas was noted.

TABLE 5

Effect of simultaneous inoculation into guinea pigs of threonine and S^o and/or S^t types of Salmonella typhimurium (average weight of animals 550 g)

COLONIAL TYPE	ORGANISMS INJECTED	THREONINE, MG INJECTED	ANIMALS	ACCUMULATIVE DEATHS BY DAYS			
				1	2	3	4
S^t	1.3×10^6	50	20	0	5	6	10
S^t	1.3×10^6	—	20	0	4	4	9
S^o	1.6×10^6	50	20	0	3	4	7
S^o	1.6×10^6	—	20	0	1	2	5
5% S^t + 95% S^o	1.7×10^6	50	20	0	5	5	10
5% S^t + 95% S^o	1.7×10^6	—	20	0	1	1	4

of threonine is most marked when a mixed population of S^o + S^t types was inoculated into guinea pigs. Under these conditions the rate of death is equal to that obtained when the S^t type alone is injected with or without threonine. This suggested that, similar to the effects observed *in vitro*, threonine produced a selective effect in favor of the more virulent, threonine-resistant S^t variant *in vivo* when mixtures were injected. Experimental evidence supported this assump-

tion, for when the heart's blood of threonine-treated guinea pigs that died after injection of 95 per cent S^o + 5 per cent S^t was plated on McConkey agar, between 5 and 50 per cent of the S^t type were detected consistently in these cultures, whereas never more than 1 per cent of the S^t type was recovered from nonthreonine-treated animals infected with the same mixture of 95 per cent S^o + 5 per cent S^t. It is noteworthy that the injection of threonine had no effect on the course of the disease in animals infected with S^t only, and only a slight effect of doubtful significance in animals infected with S^o only.

DISCUSSION

The foregoing data reveal that the increased virulence of *S. typhimurium* in guinea pigs treated with threonine can be accounted for on the basis of population changes *in vivo*, involving the establishment of more virulent, threonine-resistant variants. It is noteworthy that initial heterogenicity of the inoculum is required in order to obtain a significant threonine effect. This is in agreement with observations by Schneider (1949) who demonstrated that nutritional modifications of *Salmonella* infections can be achieved only if inocula (and hosts) are genetically heterogeneous. The reason for such differences in results, depending on whether the inoculum was homogeneous or heterogeneous, becomes quite apparent from the studies reported here. If the inoculum contains merely a few cells with virulence characteristics and growth-response (e.g., threonine-resistance) different from the majority of cells present, such variants can establish themselves rapidly under appropriate conditions which endow them with a high selective value. On the other hand, if the inoculum is homogeneous, selective forces cannot become effective immediately; the variants with increased selective value have to arise first by mutation, a process that, depending on the size of the inoculum, requires more time, since only one cell in approximately 10^6 cells will undergo spontaneous genetic changes which may lead to properties of greater selective value in the presence of altered amino acid levels. Since infection by an organism like *Salmonella* rapidly causes death even if a less virulent type like S^o is present, there is insufficient time for the establishment of a mutant arising after inoculation. Therefore, the effect of threonine or other nutritional factors has little chance to express itself prior to death. This difference between initially heterogeneous populations, permitting early selective population changes, and initially homogeneous populations, requiring time before selective changes may occur, may well be responsible for many controversial observations in the past (see Clark, 1950). Investigators who observed nutritional influences in experimental infections may have been dealing with initially heterogeneous cultures, whereas others who failed to confirm such effects may have used initially homogeneous inocula.

The data presented here are also of interest in relation to other recent results concerning the effect of metabolites on population changes *in vitro*. Goodlow, Braun, and Mika (1950, 1951) observed that the accumulation of the amino acid alanine as end product of metabolism in growing *Brucella* cultures significantly influenced the virulence of the population by favoring the establishment

of metabolite-resistant mutants with lowered virulence. Similar effects by other metabolically produced amino acids have been found since (Tucker and Braun, unpublished data). The *Salmonella* results illustrate the manner in which an amino acid can modify the course of infection by affecting population changes *in vivo*. Even though it has not been possible as yet to demonstrate conclusively whether threonine may be produced as a metabolite during growth of *Salmonella*,[2] the results of the *Brucella* and *Salmonella* studies suggest that amino acid levels in the host, produced either by the host's or the parasite's metabolism, may play a role in susceptibility and resistance through their effects on population changes involving virulence characteristics.

SUMMARY

The enhanced virulence of a *Salmonella typhimurium* culture after inoculation into guinea pigs injected simultaneously with DL-threonine was found to be due to the *in vivo* selection of a more virulent, threonine-resistant mutant that had been present in the original stock-culture. The threonine-susceptible (S^o) and the threonine-resistant (S^t) types differ in colony morphology when viewed on McConkey agar with oblique lighting, but are identical in their antigenic and in almost all of their biochemical reactions. *In vitro* S^t mutants established themselves rapidly in originally S^o populations. Similarly the proportion of S^t cells increased rapidly in threonine-treated guinea pigs infected with mixtures of S^t and S^o, whereas S^o cells predominated at time of death in similarly infected animals that received no threonine. The injection of threonine had little or no effect on death rates in animals infected with pure S^t or S^o populations. The relation of these results to problems of infection and nutrition, as well as of resistance and susceptibility, has been discussed.

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[2] During growth of *Salmonella* in certain complex media, an accumulation of threonine in the culture liquid was detected; however, free threonine was not found after growth in simple synthetic media.