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The virulence of trimethoprim-resistant thymine-requiring strains of *Salmonella*

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SUMMARY

A thymine-requiring (*thy*$^-$), trimethoprim-resistant (*tmp*^{r}) mutant isolated from the faeces of chickens experimentally infected with *Salmonella typhimurium* and treated with a mixture of trimethoprim and sulphadiazine was less virulent for chickens than the parent strain and a *thy*$^+$*tmp*^{s} revertant prepared *in vitro* from the mutant. The difference in chicken-virulence was more noticeable when the strains were administered orally than when they were administered subcutaneously. All *tmp*^{r} mutants prepared *in vitro* from four other salmonella strains were also *thy*$^-$; those tested were less virulent for chickens and mice than their parent strains. After oral infection, *thy*$^-$ salmonella organisms were found much less commonly in the alimentary tract of chickens then were *thy*$^+$ organisms. This was especially so in the caeca, the principal site of colonization of both the *thy*$^+$ and *thy*$^-$ organisms. Relatively high concentrations of thymine or related compounds were found in the contents of all regions of the alimentary tract of chickens except the caeca; the caeca usually contained low or undetectable concentrations.

The *thy*$^-$ salmonella strains would not grow on one brand of brilliant green agar because of its deficiency in thymine; their colonial appearance on other kinds of media used for isolating salmonellae from clinical material was often 'un-salmonella-like'.

INTRODUCTION

After the introduction of combinations of sulphonamides and trimethoprim for the therapy of bacterial infections in man and animals, reports have appeared on the emergence of trimethoprim-resistant (*tmp*^{r}) enterobacteria in patients so treated. These organisms may possess R factors mediating trimethoprim-resistance (Datta & Hedges, 1972; Fleming, Datta & Gruneberg, 1972) or they may be mutants which are thymine-requiring (*thy*$^-$) (Barker, Healing & Hutchison, 1972; Devriese & Hommez, 1974; Okubadejo & Maskell, 1973; Tapsall, Wilson & Harper, 1974). Several authors have reported the isolation of *thy*$^-$ organisms *in vitro* by the selective use of trimethoprim (for references, see Okubadejo & Maskell, 1973; Pinney & Smith, 1973). All the *tmp*^{r} mutants prepared *in vitro* by Pinney & Smith from enterobacteria, including one strain each of *Salmonella typhimurium*, *S. enteritidis* and enteropathogenic *Escherichia coli*, were *thy*$^-$. Because of this and the fact that some kinds of culture media are deficient in thymine or related compounds, the authors considered that *tmp*^{r}*thy*$^-$ mutants might be more common in

nature than is generally realized. We isolated such mutants of *S. typhimurium* from the faeces of chickens that had been experimentally infected with *tmp*^{s} cultures of this organism and then fed on diets containing trimethoprim/sulphadiazine (Tribrissen, Burroughs Wellcome & Co.) (Smith & Tucker, 1975). Therefore it seemed worth while comparing the virulence of these and similar mutants of other salmonella strains with that of the wild *thy*$^+$ forms from which they were derived; the results of the investigation are reported in this paper.

MATERIALS AND METHODS

Experimental animals

Light Sussex chickens from a salmonella-free flock were employed. The mice were young adults of the White Swiss breed (Tuck TO). All animals were fed *ad libitum*, the chickens on a diet consisting of ground wheat, 45%; ground maize, 45%; British white fish meal, 10%; mineral/vitamin mixture, 0·25% and the mice on Diet 41B (Oxoid).

Bacterial strains

All the salmonella strains were smooth nalidixic acid-resistant mutants (*nal*^{r}) prepared in this laboratory. When thymine-requiring (*thy*$^-$) and non-requiring (*thy*$^+$) forms of the same strain were used in infection experiments they were grown for 24 hr. at 37° C. in nutrient broth (Oxoid no. 2) to which thymine (50 μg./ml.) had been added; both forms multiplied to the same extent in this medium. This thymine-containing broth was always used for culturing *thy*$^-$ organisms.

Preparation of trimethoprim-resistant organisms in vitro

Approximately 10^9 organisms of a broth culture of a *tmp*^{s} strain were washed in phosphate buffer, pH 7·0, and inoculated on plates of synthetic medium containing trimethoprim (10 μg./ml.) and thymine (50 μg./ml.). After incubation for 48 hr., colonies that grew were purified by plating on MacConkey agar and a single colony inoculated on synthetic medium, with and without thymine; *tmp*^{r} organisms that were *thy*$^-$ failed to grow on the thymine-less medium. The synthetic medium contained (g./l.): K_2HPO_4, 7; KH_2PO_4, 3; NaCl, 5; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0·1; glucose, 5; agar 15. When used for culturing auxotrophic salmonella strains it was supplemented, at concentrations of 40 μg./ml., with the particular nutrients they required.

Isolation of thy$^+$ *revertant organisms from* thy$^-$ *cultures*

Plates of synthetic medium were flooded with washed cultures of *thy*$^-$ organisms and incubated at 37° C. for 48 hr. Colonies that grew on the plates were purified by plating and their *thy*$^+$ status confirmed.

Estimation of minimum inhibitory concentration (MIC) *of trimethoprim*

About 500 viable organisms of washed broth cultures of different salmonella strains were inoculated on sections of plates of synthetic medium containing doubly increasing amounts of trimethoprim lactate and 50 μg./ml. of thymine. The

plates were incubated at 37° C. for 48 hr. The MIC was taken as the lowest concentration that prevented visible growth.

Virulence tests with thy$^+$ *and* thy$^-$ *salmonella organisms*

Groups of one day-old chickens kept in identically constructed pens were given, as a broth culture, 5×10^8 viable organisms of either a *thy*$^+$ or a *thy*$^-$ form of the same *nal*^{r} salmonella strain directly into the crop by means of a Pasteur pipette passed down the oesophagus. Other groups were given tenfold decreasing doses of the two forms subcutaneously. The number of chickens that died on each day was recorded, the experiments being terminated 21 days after their start because deaths were uncommon after that time. The livers of most of the dead chickens were examined bacteriologically to confirm that they had died from salmonella infection. Virulence tests on mice were similar in design to those performed on chickens. They were infected by pipetting 10^9 viable organisms suspended in 0·02 ml. of broth in their mouths while they were lightly anaesthetized with ether or by giving them tenfold decreasing doses subcutaneously.

Estimations of the concentrations of salmonella organisms in faeces and alimentary contents

Faecal swabs taken from the cloaca of chickens that had been infected with *thy*$^+$ or *thy*$^-$ forms of the same *nal*^{r} salmonella strain were inoculated in a standard manner on plates of brilliant green agar (Oxoid, CM 263), modified by adding thymine (50 μg./ml.), sodium nalidixate (20 μg./ml.) and novobiocin (1 μg./ml.). The plates were incubated at 37° C. for 24 hr. Very few faecal organisms grow on this medium; the colonies of those that do can easily be differentiated visually from the colonies of the infecting salmonella strains. The degree of growth of salmonella organisms on each plate was recorded as follows: + + + + = confluent; + + + = almost confluent; + + = partly confluent; + = numerous mainly discrete colonies; ± = numerous discrete colonies; 50, 5, 1 = approximately 50 colonies, 5 colonies and 1 colony respectively. In addition, the faecal swabs were placed in thymine-supplemented selenite broth and, after incubation at 37° C. for 24 hr., subcultured on brilliant green agar.

In some experiments weighed amounts of the contents of the alimentary tracts of chickens and mice were diluted tenfold in phosphate buffer, pH 7·0, and the numbers of organisms of the infecting strain present counted on the modified brilliant green agar by the method of Miles & Misra (1938).

Because *thy*$^-$ organisms can revert to *thy*$^+$ *in vivo*, at least one colony from each plate used for culturing alimentary contents or organs of animals that had been infected with *thy*$^-$ salmonella strains was subcultured on the synthetic medium; colonies that grew on this medium were recorded as *thy*$^+$ revertants.

In some experiments, animals were infected with mixtures of equal numbers of *thy*$^+$ and *thy*$^-$ organisms of the same salmonella strain. To facilitate the enumeration of each kind of organism in the alimentary contents, the *thy*$^+$ ones were used as spectinomycin-resistant mutants (*spc*^{r}) and the *thy*$^-$ ones as streptomycin-resistant mutants (*str*^{r}), the bacterial counts being performed in duplicate on the

modified brilliant green agar with spectinomycin (40 μg./ml.) or streptomycin (40 μg./ml.) added.

The examination of biological materials for thymine or related substances

Measured amounts of food, contents of different parts of the alimentary tract and internal organs of chickens, after grinding, where necessary, with sterile sand, were mixed with the least amount of distilled water that would permit separation into a fluid and a solid phase after centrifuging in an M.S.E. Major centrifuge for 1 hr. at 4000 rev./min. After their pH was adjusted to approximately 7·0 the supernatant fluids were either boiled for 15 min. or filtered through a Seitz E.K. Special filter. The filtrates were concentrated by freeze-drying and made up to the original volumes of the materials from which they were obtained. In some cases, the original uncentrifuged suspensions were placed in Viscin seamless tubing and dialysed against a small volume of distilled water. The effusate and the boiled and Seitz-filtered fluids were then added to equal volumes of melted double-strength synthetic medium at 60° C. and poured into Petri dishes. After drying, the plates were inoculated with washed suspensions of the *thy*$^+$ and *thy*$^-$ forms of the same *S. typhimurium* strain and incubated at 37° C. for 48 hr. The degree of the growth on each plate and on plates of synthetic medium containing different amounts of thymine was then compared. Thymine content was also estimated by a modification of the agar well diffusion method used for antibiotic assay, the extracts being placed in wells made with a cork-borer in plates of synthetic medium lightly seeded with a *thy*$^-$ salmonella culture. The plates were incubated at 37° C. for 48 hr. and the width of the zones of bacterial growth around the holes measured. The wells in some plates were filled with untreated alimentary contents; to control bacterial contaminants, a *thy*$^-$ strain that was *spc*^{r} in addition to *nal*^{r} was used as the assay culture and spectinomycin (20 μg./ml.) and sodium nalidixate (20 μg./ml.) were incorporated in the culture medium.

RESULTS

Characteristics of thy$^-$ *salmonella strains*

The *tmp*^{r}*thy*$^-$ salmonella strain employed in most of the studies was isolated from the faeces of a chicken that had been infected orally with a wild *Salmonella typhimurium* strain, No. 98, of phage type 14 and then fed on a diet containing trimethoprim (20 mg./kg.) and sulphadiazine (100 mg./kg.). The other *tmp*^{r}*thy*$^-$ strains studied were prepared by making a *S. typhimurium* strain, 5235 of phage type 29, provided by Professor E. S. Anderson, a *S. dublin* strain, a *S. choleraesuis* strain and a *S. gallinarum* strain resistant to trimethoprim *in vitro*. Their mutation rates to resistance varied from 1 in 10^9 for the *S. gallinarum* strain to 1 in 10^7 for the *S. typhimurium* strain. All of 36 *tmp*^{r} cultures prepared from the four salmonella strains were *thy*$^-$. The minimum inhibitory concentration (MIC) of trimethoprim for the five different *tmp*^{r}*thy*$^-$ salmonella strains varied from 40–300 μg./ml.; the MIC of the strains from which they were derived varied from 0·5–1·0 μg./ml. *Thy*$^+$ revertants, which were *tmp*^{s}, were obtained from the *thy*$^-$ *S. typhimurium* 98 and

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Table 1. *The mortality rate in groups of chickens given* thy$^+$ *or* thy$^-$ *forms of different salmonella strains orally*

Strain	Form	No. of chickens infected	% that died
S. typhimurium 98	*thy*$^-$	112	0
	thy$^+$	112	48
	thy$^+$(*thy*$^-$)*	62	47
S. typhimurium 5235	*thy*$^-$	40	5
	thy$^+$	40	50
S. dublin	*thy*$^-$	40	5
	thy$^+$	40	25
S. choleraesuis	*thy*$^-$	40	0
	thy$^+$	40	13
S. gallinarum	*thy*$^-$	40	3
	thy$^+$	40	100

* A *thy*$^+$ revertant prepared in the laboratory from the *thy*$^-$ strain. Each chicken was given 5×10^8 viable organisms when 1 day old.

Table 2. *The mortality rate in groups of* 25 *chickens given* thy$^-$ *and* thy$^+$ *forms of* Salmonella typhimurium 98 *subcutaneously*

Dose (viable organisms)	No. that died after being given the *thy*$^-$ form	No. that died after being given the *thy*$^+$ form
5×10^8	20	25
5×10^7	20	24
5×10^6	9	19
5×10^5	6	10
5×10^4	1	3

The chickens were 1 day old when they were infected.

5235 strains, their reversion rates being in the region of 1 in 10^{10}. It was not possible to obtain revertants from the *thy*$^-$ *S. dublin*, *choleraesuis* and *gallinarum* strains.

Unless supplemented by thymine, brilliant green agar (Oxoid, CM 263) and diagnostic sensitivity test agar (Oxoid, CM 261) would not support the growth of the five *thy*$^-$ salmonella strains; optimal growth on the synthetic medium required the addition of about 25 μg./ml. of thymine. The *thy*$^-$ strains grew on brilliant green agar (Difco, B 285), blood agar base (Oxoid, CM 55), tryptose agar (Difco, B 64), MacConkey agar (Oxoid, CM 7), deoxycholate citrate agar (Oxoid, CM 35) and Wilson and Blair's medium (Oxoid, CM 201) but their colonies were usually unlike salmonella colonies, being smaller, flatter, and clearer than those of the corresponding *thy*$^+$ strains. When thymine was added to all these media, colonies of *thy*$^-$ strains resembled those of *thy*$^+$ strains. The *thy*$^-$ strains grew more poorly in nutrient broth No. 2 (Oxoid, CM 67) than the *thy*$^+$ strains, the colony count after incubation at 37° C. for 24 hr. being about one-third that of the *thy*$^+$ strains. The colony count of the *thy*$^-$ strains in selenite broth (Oxoid, CM 39)

incubated at 37° C. for 24 hr. was about half that of the *thy*$^+$ strains; the *S. choleraesuis* strain was not tested because of the known inability of strains of this serotype to grow in selenite medium.

The virulence of thy$^+$ *and* thy$^-$ Salmonella *strains for chickens*

The mortality rates that occurred in groups of chickens infected orally with *thy*$^+$ or *thy*$^-$ forms of the five salmonella strains are shown in Table 1. The *thy*$^-$ forms of all five strains were much less virulent than the corresponding *thy*$^+$ forms. The mortality rate in the group given the *thy*$^+$ revertant prepared in the laboratory from the *thy*$^-$ form of *S. typhimurium* 98 was the same as that in the group given the wild *thy*$^+$ form from which the *thy*$^-$ form had originated *in vivo*.

The results of infecting groups of chickens subcutaneously with different doses of the *thy*$^+$ or *thy*$^-$ forms of *S. typhimurium* 98 are shown in Table 2. Although the mortality rate in the group given 5×10^6 viable organisms of the *thy*$^+$ form was significantly higher than in the group given the same dose of the *thy*$^-$ form ($0{\cdot}001 < P < 0{\cdot}01$), the difference between the two forms was much less obvious than when they were given orally. When four groups of ten 1-day-old chickens were injected subcutaneously with 10^8 viable organisms of the *thy*$^+$ forms of *S. typhimurium* 5235, the *S. dublin*, the *S. choleraesuis* and the *S. gallinarum* strains, all 40 died. So did all ten chickens in each of two groups injected with the same dose of the *thy*$^-$ forms of *S. typhimurium* 5235 or of the *S. dublin* strain; only four of ten injected with the *thy*$^-$ form of the *S. choleraesuis* strain and two of the ten injected with the *thy*$^-$ form of the *S. gallinarum* strain died.

The survival of thy$^+$ *and* thy$^-$ *forms of the salmonella strains in the alimentary tract of chickens*

The results of estimating at different times after infection the concentrations of salmonella organisms in the faeces of the chickens used in the oral virulence studies (Table 1) are summarized in Table 3; only those for 50 of the chickens infected with each of the *thy*$^+$ and *thy*$^-$ forms of *S. typhimurium* 98 are quoted. Much higher concentrations of salmonella organisms were found in the faeces of the chickens infected with the *thy*$^+$ forms than in the faeces of the chickens infected with the *thy*$^-$ forms of all five salmonella strains; the chickens given the *thy*$^+$ forms also remained faecal excreters much longer than those given the *thy*$^-$ forms did. *Thy*$^+$ revertants were isolated from the faeces of some of the chickens given *thy*$^-$ forms of the two *S. typhimurium* strains; in these chickens the *thy*$^+$ organisms soon dominated the *thy*$^-$ ones.

When tenfold falling concentrations of the *thy*$^-$ and *thy*$^+$ forms of *S. typhimurium* 98 were given orally to groups of chickens, the difference between the two forms in their ability to colonize the alimentary tract was greatest at the 5×10^6 dose level. The numbers of organisms found in the *thy*$^-$ groups given 5×10^7 or 5×10^8 viable organisms, although low, was sufficiently high to give rise to *thy*$^+$ revertants which within ten days of the commencement of the experiment became more common in these groups than the *thy*$^-$ organisms themselves.

Counting the numbers of viable salmonella organisms in the contents of different

Table 3. *The faecal excretion of salmonella organisms by groups of chickens that had been infected orally with* thy⁻ *or* thy⁺ *forms of different salmonella strains*

Infecting strain	Time after infection (days)	% of chickens whose faeces had the following concentrations of salmonella organisms after they were infected with the *thy⁻* form				*thy⁺* form			
		> +	> 50	D	T	> +	> 50	D	T
S. typhimurium 98	4	0	39	61	78	35	88	100	100
	11	8	15	21	69	60	94	97	97
	18	2	6	8	24	19	65	85	88
	25	0 (2)	0 (4)	7 (40)	12 (65)	12	36	49	88
	32	0	0	2 (8)	4 (14)	4	24	32	48
	39	0	0	0	2	5	5	10	15
S. typhimurium 5235	2	53	93	95	98	87	98	98	98
	5	8 (6)	25 (14)	46 (14)	42 (14)	70	100	100	100
	7	3 (6)	12 (6)	12 (6)	26 (6)	75	100	100	100
	9	8 (3)	27 (3)	48 (3)	48 (3)	77	100	100	100
	12	8 (3)	28 (8)	36 (11)	59 (11)	48	100	100	100
	14	0 (3)	14 (3)	20 (3)	54 (3)	55	100	100	100
	21	3 (3)	13 (11)	18 (25)	35 (31)	15	60	95	95
S. dublin	2	26	87	92	92	75	98	100	100
	5	3	12	24	35	68	95	100	100
	7	0	0	0	0	55	91	100	100
	9	0	0	0	0	32	74	97	97
	12	0	0	0	0	14	55	86	90
	14	0	0	0	0	10	10	62	83
	21	0	0	0	0	8	32	64	80
S. choleraesuis	2	25	65	88	88	67	95	98	98
	5	13	19	29	29	57	88	94	94
	7	0	0	0	0	18	48	91	91
	9	0	0	0	0	18	61	91	91
	12	0	0	0	0	3	29	84	84
	14	0	3	6	6	7	40	70	70
	21	0	0	0	0	28	38	76	76
S. gallinarum	2	3	3	3	16	60	93	95	95
	5	0	10	51	51	67	84	93	93
	7	8	15	11	11				
	9	24	43	76	76				
	12	0	32	65	65				
	14	0	19	58	58				
	21	0	12	49	55				

The chickens were those employed in virulence studies (Table 1). The faeces of all survivors were examined on each occasion; the whole *S. gallinarum thy⁺* group died within 6 days of infection. The figures in parentheses refer to chickens in which only *thy⁺* revertant organisms were found.

T = isolated by selenite enrichment or direct culture; D = isolated by direct culture; 50 = 50 colonies on the culture plate; + = the culture plate was covered by colonies that were mainly discrete.

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Table 4. *Concentration of* thy$^+$ *and* thy$^-$ Salmonella typhimurium 98 *organisms in the alimentary tract of chickens at different times after they were given equal numbers of these two forms orally*

Time after infection (days)	No. of chickens examined	No. of organisms, $\times 10^{-3}$, per g. of contents of: Crop *thy*$^-$	Crop *thy*$^+$	Small intestine *thy*$^-$	Small intestine *thy*$^+$	caeca *thy*$^-$	caeca *thy*$^+$	cloaca *thy*$^-$	cloaca *thy*$^+$
1/4	5	40 (0–70)	30 (0–100)	7 (0·6–20)	1 (0–25)	1,500 (1,500–50,000)	2,500 (1,500–50,000)	50 (25–500)	35 (10–800)
1/2	5	0·3 (0–5)	0 (0–1)	0·1 (0–2)	0·1 (0–1)	400 (50–3,000)	300 (8–600)	1 (0–400)	0·3 (0–80)
3/4	5	0 (0–0·2)	0 (0–0)	0 (0–0)	0 (0–0)	5,000 (250–30,000)	4,000 (200–5,000)	0 (0–400)	0 (0–50)
1	11	0 (0–5)	0 (0–50)	0 (0–0)	0 (0–0·2)	400 (0·6–2000)	4,000 (0·6–750,000)	0 (0–500)	0 (0–7,500)
2	6	0 (0–0)	0 (0–50)	0 (0–0)	0·5 (0–1)	2 (0–7)	50,000 (100–250,000)	0 (0–0)	25 (0·6–70)
3	6	0 (0–0)	0 (0–50)	0 (0–0)	0·4 (0–5)	4 (0–20)	300 (0·4–600,000)	0 (0–0)	35 (0–300)
4	6	0 (0–0)	0 (0–50)	0 (0–0)	2 (0–40)	0·1 (0–1)	12,000 (0–300,000)	0 (0–0)	0·2 (0–80)
7–9	18	0 (0–0)	0 (0–20)	0 (0–0)	0 (0–8)	0 (0–6)	350 (0–25,000)	0 (0–0)	9 (0–700)
10–11	12	0 (0–0)	0 (0–0)	0 (0–0)	0·3 (0–1000)	0·5 (0–1)	200 (0–300,000)	0 (0–2)	9 (0–30)
14–15	12	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0·2)	0·5 (0–5000)	0 (0–0)	0 (0–2)

The median count is given followed by, in parentheses, the range. The chickens were infected when 4 days old with $2{\cdot}5 \times 10^8$ viable organisms of each form. The *thy*$^-$ organisms were *nal*^{r}*str*^{r} mutants and the *thy*$^+$ organisms were *nal*^{r}*spc*^{r} mutants; they were differentiated by counting in duplicate on media containing sodium nalidixate and streptomycin and sodium nalidixate and spectinomycin.

Table 5. *The mortality rate in groups of* 18 *mice given* thy⁻ *and* thy⁺ *forms of* Salmonella typhimurium 5235 *subcutaneously*

Dose (viable organisms)	No. that died after being given the *thy*⁻ form	No. that died after being given the *thy*⁺ form
5×10^8	4	15
5×10^7	0	10
5×10^6	0	4
5×10^5	0	2

regions of the alimentary tract of chickens given 5×10^8 *thy*⁺ or *thy*⁻ organisms of *S. typhimurium* 98 orally when 1-day-old and killed in groups of five 1, 2 or 4 days later revealed that much lower concentrations of *thy*⁻ than *thy*⁺ organisms were present, not only in the faeces, but also in the crop, upper and lower small intestine and, especially, the caeca. Organisms were also found in the liver of all 15 chickens given the *thy*⁻ form but in lower concentrations than in the liver of the chickens given the *thy*⁺ form. Groups of five chickens were also examined 5 or 7 days after infection. Only *thy*⁺ revertants were found in the alimentary tract and liver of the ten chickens given the *thy*⁻ organisms, their concentrations being similar to those found in the chicken given the *thy*⁺ organisms.

The results of estimating the numbers of salmonella organisms in the contents of different regions of the alimentary tract of chickens given equal mixtures of *nal*ʳ*spc*ʳ*thy*⁺ and *nal*ʳ*str*ʳ*thy*⁻ organisms of *S. typhimurium* 98 are summarized in Table 4. Until 1 day after infection there was little difference between the concentrations of *thy*⁺ and *thy*⁻ organisms in the contents of each of the organs examined. Thereafter, the *thy*⁻ organisms were greatly outnumbered by the *thy*⁺ ones, particularly in the caeca, the region in which both forms were by far the most numerous.

The virulence of thy⁻ *and* thy⁺ *forms of* Salmonella typhimurium *for mice*

When 25 mice were infected orally with 10^9 viable organisms of the *thy*⁻ form of *S. typhimurium* 5235, two died; the corresponding figure for 25 mice similarly infected with the *thy*⁺ form was seven. The *S. typhimurium* 98 strains were not tested orally because the *thy*⁺ parent strain was not lethal for mice by this route. The results of comparing the virulence for mice of the *thy*⁺ and *thy*⁻ forms of strain 5235 subcutaneously are summarized in Table 5. Although the virulence of the *thy*⁺ form for the particular strain of mice used was not high it was greater than that of the *thy*⁻ form. Employing doses of 5×10^8 viable organisms, all of five mice inoculated subcutaneously with the *thy*⁺ form of *S. typhimurium* 98 and one of five mice inoculated with a similar dose of the *thy*⁻ form of this strain died; none of five mice died when given 5×10^7 viable organisms of the *thy*⁺ or *thy*⁻ form. Identical results were obtained when the experiment was repeated with the *thy*⁺ and *thy*⁻ forms of the *S. dublin* strain.

Table 6. *The isolation of* thy⁻ *and* thy⁺ *forms of* Salmonella typhimurium *strain* 98 *and* 5235 *from the caecal contents and livers of mice after oral administration*

Strain	Form	No. of mice	% of mice harbouring* the form in their: Caecal contents D	Caecal contents T	Livers D	Livers T
98	*thy*⁻	40	8	33	3	3
	thy⁺	40	35	73	8	38
5235	*thy*⁻	30	20	50	0	46
	thy⁺	30	73	100	46	93

* Five days after they were given 10^9 viable organisms of strains 98 or 17 days after they were given a similar dose of strain 5235.

T = isolated by selenite enrichment or direct culture; D = isolated by direct culture.

The survival of thy⁻ *and* thy⁺ *forms of salmonella strains in mice*

The results of examining the caecal contents and livers of mice five or 17 days after they had been infected orally with *thy*⁻ and *thy*⁺ forms of *S. typhimurium* 98 or 5235 are summarized in Table 6. Although *thy*⁻ organisms were found less commonly than *thy*⁺ ones, the difference between the two forms, especially in ability to colonize the caeca, was less obvious than it was in chickens.

The presence of thymine or related compounds in extracts of tissues or alimentary contents

Synthetic medium containing extracts of liver, spleen, muscle, small intestinal wall, caecal wall and cloacal wall and blood and serum of chickens supported the growth of the *thy*⁻ form of *S. typhimurium* 98. So did extracts of their food, their faeces and the contents of their crops and small intestines. The extracts of the small intestinal contents still supported growth when diluted 16 times and so did the faecal extracts when diluted four times. Extracts of caecal contents from some chickens supported growth but others failed to do so. All the chicken caecal contents examined contained much less thymine-like substances than did small intestinal contents. This was especially noticeable when the contents of both organs were compared by the agar well diffusion method, wide zones of growth of the *thy*⁻ strain occurring around the wells filled with different specimens of small intestinal contents and narrow zones or no zones occurring around the wells filled with specimens of caecal contents. Relatively wide zones surrounded wells filled with mouse caecal contents.

DISCUSSION

When administered subcutaneously and, especially, orally, the *thy*⁻ form of *Salmonella typhimurium* 98 was less virulent for chickens than the *thy*⁺ form from which it had arisen *in vivo* during trimethoprim therapy. The fact that the *thy*⁺ revertant prepared *in vitro* from the *thy*⁻ form exhibited the same degree of virulence as the *thy*⁺ parent form confirmed that thymine synthesis was importantly

connected with the difference in virulence between the two forms. A considerable difference in virulence for chickens was also noted between the *thy*$^+$ forms of the *S. typhimurium* 5235, *S. dublin*, *S. choleraesuis* and *S. gallinarum* strains and the *thy*$^-$ forms prepared from them *in vitro*. An important bacteriological feature of the oral virulence tests was the very low concentrations of salmonella organisms in the alimentary tract of the chickens given *thy*$^-$ organisms (unless reversion to the *thy*$^+$ state occurred) and the comparatively high concentrations in the alimentary tract of those given *thy*$^+$ organisms. This difference was particularly noticeable in the caecum, the organ shown in the case of *S. typhimurium* 98 to be the main site of colonization of both *thy*$^+$ and *thy*$^-$ organisms, and it correlates with the finding that the caecal content of the chickens used in these experiments contained little or no thymine. Much more was found in the contents of other parts of the alimentary tract. Sufficient concentration to support the growth of *thy*$^-$ organisms, too, was found in the tissues of chickens. These concentrations, however, may not always be readily available or usable in the living animal and this may permit the difference in thymine-requirement between the two salmonella forms to express itself in terms of virulence. The situation, too, may be accentuated in orally infected chickens, but perhaps not in orally infected mice, by the relative or absolute deficiency of thymine in their caeca, the principal colonization site of salmonella organisms. Lack of substrate in the peritoneal cavity has been reported as the cause of reduced virulence in purine-requiring mutants of *Salmonella typhi*, the virulence of these strains being partly or completely restored to that of their parent strain or revertant strains by injecting the purine intraperitoneally at the same time as the infecting organisms (Bacon, Burrows & Yates, 1951; Formal, Baron & Spilman, 1954).

The failure of the *thy*$^-$ strains to grow on Oxoid brilliant green agar, due to thymine deficiency, reinforces the opinion of Pinney & Smith (1973) that some *thy*$^-$ mutants may go undetected during the examination of clinical material. Although the strains grew on other media commonly employed for isolating salmonellas, their colonial appearance on some might have been sufficiently unlike salmonellas for them to be overlooked under ordinary circumstances. The results of the present study, however, suggest that in view of their reduced virulence and colonizing ability the emergence of *tmp*^{r}*thy*$^-$ salmonellas during trimethoprim therapy, although important, may not be quite as serious as would be imagined at first sight.

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