GROWTH OF *SALMONELLA TYPHI* AND CERTAIN OTHER MEMBERS OF THE *SALMONELLA* GROUP IN MILK AND BUTTER STORED AT ATMOSPHERIC TEMPERATURES

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In estimating the significance of chance contamination in the causation of milk-borne epidemics of typhoid and paratyphoid fever and of *Salmonella* food poisoning, it is desirable to have exact knowledge of what occurs when various *Salmonella* organisms are introduced into milk held under ordinary conditions of commercial and household storage.

As long ago as 1896, Cautley reported that *S. typhi* could survive in milk stored for several days under household conditions. Pfuhl (1902) reported that this organism could live for 13 days in milk and for 24 days in butter. Bruck (1903) found that it survived in cream for at least 10 days after separation and could be isolated from contaminated butter after 27 days. Eyre (1904) found that if small numbers were added to milk, they multiplied about a hundredfold in 24 hr. Wade *et al.* (1928) found that *S. typhi* survived in Cheddar cheese manufactured from contaminated milk for at least 36 days.

Andresen (1932) found that paratyphoid bacilli (type not specified) also multiplied in raw milk stored at atmospheric temperature. At 14° C. he noted fourfold multiplication in the course of 24–28 hr. storage, while in previously boiled milk stored under similar conditions one hundredfold multiplication occurred. Kaiser (1921) reported the survival of *S. paratyphi* B in yoghurt (sour milk) for periods varying from 12 to 72 hr.

The object of the present investigation, therefore, has been to attempt to confirm the findings of previous workers with regard to *S. typhi*, paying special attention to the initial degree of contamination with this organism, and to compare the behaviour of stock strains with that of very recently isolated ones. The behaviour of *S. paratyphi* B, *S. dublin*, *S. cholerae-suis*, *S. typhi-murium*, *S. enteritidis*, and *S. bovis-morbificans* has been studied along similar lines, since these species are known to cause food poisoning in man and are liable to contaminate milk. In addition, a few experiments have been carried out to test the viability of all these organisms in butter stored at room and refrigeration temperatures.

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Plan of investigation

When this investigation was originally planned it was intended to study the behaviour of *S. typhi* in all commercial grades of milk stored at temperatures ranging between 15 and 22° C., and a number of preliminary experiments were carried out along these lines. From the results of these experiments (see Table II), which coincided with the findings of other workers reported in the literature, it became evident that the important point was whether or not the various members of the *Salmonella* were so resistant to the bacteristatic effect of very fresh raw milk as to be able to multiply rapidly in it during storage at low atmospheric temperatures such as 15° C. This aspect of the problem was therefore examined in detail and for the purpose fresh milk was obtained from individual cows and used as soon as possible after withdrawal. The behaviour of the undermentioned strains of *Salmonella* in such milk during storage at 15° C. was ascertained:

S. typhi, Strains Nos. 1 and 2	Old stock cultures
S. typhi, Strains Nos. 3 and 4	Very recently isolated strains
S. paratyphi B	Recently isolated strain
S. dublin, Strain No. 1	Old stock culture
S. dublin, Strain No. 2	Recently isolated strain
S. cholerae-suis	,, ,,
S. typhi-murium	,, ,,
S. enteritidis	,, ,,
S. bovis-morbificans	,, ,,

Technique

Milk

As a preliminary step it was necessary to develop a technique for recognizing and counting these organisms in milk. More accurate colony counts are obtained from deep than from surface plates because of the larger initial inoculum which may be used for deep culture and the better distribution of colonies obtained. Many selective and indicator media were therefore examined in the hope of finding one in which *S. typhi* produced distinctive colonies in deep culture, but no success was obtained in this direction and the necessity for developing a satisfactory technique for surface culture was recognized. MacConkey agar, which is cheap and easy to prepare, was ultimately chosen as the most suitable medium for surface culture, as it inhibited most of the normal saprophytes of milk and allowed *Salmonella* to develop distinctive colonies with only slight reduction of vitality. The influence of the MacConkey medium upon vitality was tested by inoculating sterile milk with *S. typhi* and then making counts on both plain and MacConkey agar. The figures are recorded in Table I and show that whilst the number of colonies seen on MacConkey agar was smaller than on plain agar, this

difference was not significant when compared with the degree of multiplication on which later conclusions are based.

The method of seeding the milk under investigation and of making the subsequent dilutions for plating was identical with that previously described (Pullinger & Kemp, 1937) and is indicated in the accompanying diagram. The procedure consisted of mixing 1 c.c. amounts of tenfold dilutions in tap water of a serum broth culture of the *Salmonella* under test with 9 c.c. amounts of milk. *Salmonella* and total bacterial counts were made immediately, and after intervals of storage, by preparing serial tenfold dilutions of each sample of milk in sterile tap water and inoculating plates from these dilutions.

Table I. *Comparison of colony counts of* S. typhi *from MacConkey agar and plain agar*

Exp.	Medium	Plate counts 1	2	3	4	5	Average counts
A	MacConkey	21	32	25	24	27	25·8
	Plain agar	28	29	27	31	34	29·8
B	MacConkey	39	35	30	33	29	33·2
	Plain agar	29	43	38	48	—	37·5
C	MacConkey	29	49	43	40	38	39·8
	Plain agar	54	53	59	54	45	53·0
D	MacConkey	40	45	48	35	—	42·0
	Plain agar	47	38	46	43	42	43·2

To stop colonies from spreading on the surface of the MacConkey agar and to prevent the formation of daughter colonies around the original ones, it is essential that the surface of the medium to be inoculated be dry and the lid of the Petri dish free from water of condensation. Considerable difficulty was encountered in drying sufficiently quickly the large numbers of plates needed, as it was found that prolonged drying tended to spoil the nutritive value of the medium. The procedure finally devised was to pour plates and leave them to harden on the bench overnight; the following morning they were stacked right way up in couples on a hot plate,[1] when the surface of the medium dried rapidly, water vapour collecting in large drops on the lids of the Petri dishes. At intervals the lids were removed one by one and the drops of water were shaken out, and by the afternoon the plates were in a suitable condition for inoculation. The MacConkey medium did not become contaminated as a result of shaking the lids.

When sowing the plates, a volume of 0·2 c.c. was found to be a convenient sized inoculum, and five plates were inoculated from each dilution expected to give suitable counts. The inoculum was spread evenly over the surface of the medium by means of a thin glass spreader, which was resterilized in a pot of boiling water before use. A spreader made by bending a Pasteur pipette was found to be suitable as it dried and cooled almost instantaneously. Spreading was carried out on each set of five plates immediately after inoculation. Total bacterial counts were made by inoculating 1 c.c. amounts of

[1] The top of a hot water radiator served this purpose satisfactorily.

dilutions into poured agar plates. *Salmonella* colony counts were made after plates had been incubated for 24 hr. at 37° C., while total bacterial colony counts in plain agar were made after 48 hr. With very little practice it was easy to distinguish *Salmonella* colonies, as none of the saprophytes of milk produced similar growth; with *S. typhi*, however, for confirmatory purposes agglutination tests were carried out. For this purpose 30–50 colonies considered to be *S. typhi* were picked into 1 c.c. amounts of serum broth and incubated at 37° C. for 24 hr. The growth was then killed by adding 0·25 % formalin

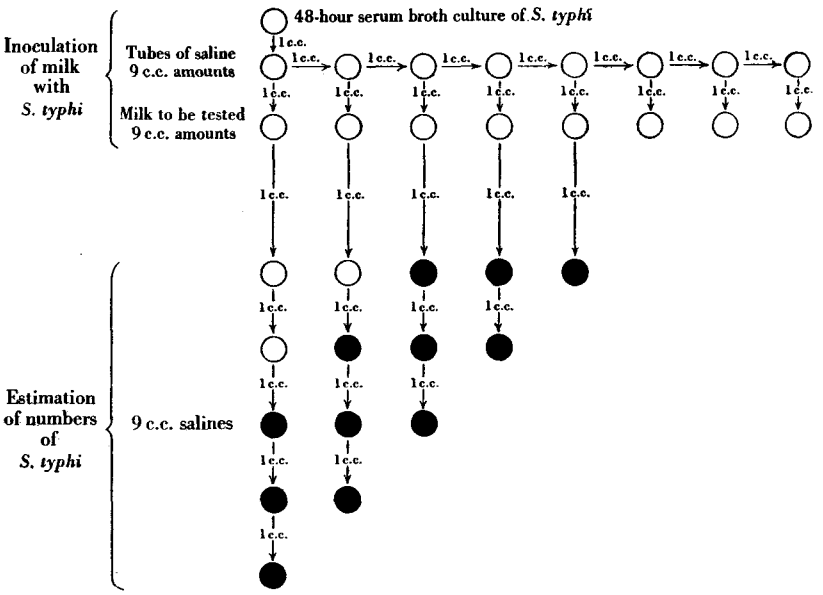

Diagram 1.

to each tube and heating for an hour at 55° C. A suitable quantity of dilute *S. typhi* antiserum was next added, and the tubes were examined for agglutination after 2 and 4 hr. incubation in a water bath at 55° C. Growth from all colonies picked as *S. typhi* from MacConkey agar invariably showed agglutination with the specific antiserum; this agglutination was generally of the "H" type, but sometimes pure "O" agglutination was seen. It was concluded, therefore, that the visual recognition of these colonies was adequate and the confirmatory agglutination test was only occasionally carried out when the other species of *Salmonella* were examined.

Butter

Since facilities were not available for manufacturing butter from cream that was already contaminated with *Salmonella*, the organism was introduced by taking a trace of bacterial growth from an agar culture and working this

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thoroughly into the butter in a sterile mortar with the aid of a pestle and palette knife. The butter was then packed in sterile test tubes and stored at either 15 or 3° C. Various grades, including home, empire, foreign, salted and unsalted butter were examined. At various intervals of storage, tests for the survival of *Salmonella* were made by digging a small piece of butter from the depths of the mass with a strong platinum spade and smearing this over MacConkey agar. Warming the spade slightly facilitated the spreading of the butter. No attempt was made to count colonies of *Salmonella*, since any figures thus obtained would have been meaningless, but the presence of *Salmonella* was noted and their identity was occasionally confirmed by agglutination.

RESULTS

Preliminary experiment with S. typhi

Results of this are summarized in Table II, from which it will be seen that after an initial lag period lasting in certain cases up to 24 hr. *S. typhi* multiplied readily in sterilized and pasteurized milk and also in fresh milk which had been stored some hours prior to contamination. As a result of these findings, attention was focused upon the behaviour of this organism when added to absolutely fresh raw milk in which the natural bacteristatic factor was still active.

Table II. *Preliminary investigation of the behaviour of an old stock strain of* S. typhi *in various classes of milk*

Class of milk	Storage temp. ° C.	Result of seeding with *S. typhi*
Sterilized	18	Multiplication beginning within 24 hr.
Pasteurized (laboratory)	18	,, ,, ,, 24 hr.
Pasteurized (commercial)	18	,, ,, ,, 24 hr.
	15	,, ,, ,, 48 hr.
Fresh raw, stored at room temp. for 4 hr. prior to seeding	18	,, ,, ,, 48 hr.
	22	,, ,, ,, 24 hr.
Fresh raw, stored in refrigerator for 4 hr. prior to seeding	18	,, ,, ,, 24 hr.

Survival of S. typhi *in fresh raw milk*

Results are summarized in Table III, from which it will be seen that, whilst this organism always multiplied in fresh raw milk at atmospheric temperatures, there were surprising variations in the rate of growth of the different strains. The old stock strains, when stored at 15° C., showed a long lag period lasting 48 hr. or more. With the newly isolated strains, on the other hand, the initial lag period was very short, and significant multiplication occurred within 24 hr. of storage. Storage at 18° C. produced no very striking increase in the rate of growth as compared with that observed at 15° C.

As regards the effect of the size of the initial inoculum of *S. typhi* in milk on the subsequent behaviour of the organisms, it is evident that multiplication occurred whether very few or very many organisms were added to the milk,

Table III. *Growth of* S. typhi *in fresh raw milk stored at atmospheric temperatures*

No. of exp.	No. of cow	Strain of *S. typhi*	Temp. of storage ° C.	Initial inoc. of *S. typhi* (orgs. per c.c.)	Count of *S. typhi* after storage	
					24 hrs.	48 hrs.
1	1	Old 1	15	2,095,000	846,000	390,000
				12,460	6,560	5,500
				166	136	138
2	1	Old 2	15	772,000	344,000	660,000
				7,560	6,020	22,870
				103	120	205
3	1	New 3	15	274,000	520,000	1,578,000
				2,580	11,900	30,000
				242	1,116	3,000
4	1	New 3	15	273,000	572,000	1,664,000
				2,400	8,140	20,000
				307	772	2,000
5	2	New 3	15	474,000	1,044,000	1,420,000
				4,100	13,840	20,000
				316	1,563	2,000
6	1	New 4	15	270,000	414,000	1,085,000
				2,520	11,320	>20,000
				64	140	392
7	1	New 4	15	200,000	574,000	1,600,000
				2,850	5,760	>20,000
				36	78	298
8	2	New 4	15	376,000	655,000	3,500,000
				31,800	115,800	904,000
				248	1,262	2,500
9	1	Old 2	18	426,000	784,000	>2,000,000
				4,500	13,670	>200,000
				80	100	252
10	1	New 4	18	536,000	1,056,000	2,364,000
				5,380	17,940	>200,000
				84	120	228

but the most rapid increases seemed to occur when the initial inoculum was intermediate in size.

Survival of other Salmonella *in fresh raw milk*

Results are summarized in Table IV, from which it will be seen that all species examined, viz., *S. dublin*, *cholerae-suis*, *paratyphi* B, *typhi-murium*, *enteritidis* and *bovis-morbificans* multiplied in milk stored at 15° C. as readily as did *S. typhi*.

Survival of Salmonella *in butter*

Results are summarized in Tables V and VI, from which it will be seen that *S. typhi* survived for at least 80 days in all classes of butter when stored at both room (15° C.) and refrigeration (3° C.) temperatures. At 3° C. the organism still survived on the 108th day, though in diminishing numbers, but on the 126th it could no longer be isolated. In two out of four samples stored at 15° C. *S. typhi* was isolated on the 80th day, but by the 126th day the butter was so rancid that contaminants completely overgrew all the culture plates.

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Table IV. *Growth of various species of* Salmonella *in fresh raw milk stored at* 15° C.

No. of exp.	No. of cow	Species of *Salmonella*	Initial inoc. of *Salmonella* (orgs. per c.c.)	Count of *Salmonella* after storage	
				24 hrs.	48 hrs.
1	1	*enteritidis*	705,000	2,500,000	10,670,000
			6,400	>200,000	>2,000,000
			48	264	1,126
2	2	*enteritidis*	622,000	>2,000,000	15,000,000
			7,600	>20,000	>200,000
			60	546	>2,000
3	1	*bovis-morbificans*	600,000	1,916,000	12,560,000
			7,240	10,020	105,000
			88	132	196
4	2	*bovis-morbificans*	248,000	1,500,000	15,000,000
			3,880	25,000	>250,000
			338	1,500	>25,000
5	1	*typhi-murium*	378,000	720,000	>2,000,000
			5,200	4,980	>20,000
			38	22	134
6	2	*typhi-murium*	326,000	>2,000,000	>20,000,000
			2,470	>35,000	>350,000
			40	400	>2,500
7	1	*cholerae-suis*	724,000	920,000	>2,000,000
			5,240	6,360	>20,000
			96	42	252
8	2	*cholerae-suis*	334,000	994,000	5,800,000
			3,860	4,820	>200,000
			30	46	460
9	1	*paratyphi* B	210,000	3,000,000	25,000,000
			2,080	25,000	200,000
			82	166	2,000
10	2	*paratyphi* B	362,000	1,458,000	13,370,000
			3,220	14,950	>200,000
			50	48	950
11	1	*dublin*	1,154,000	2,460,000	>20,000,000
			1,145	2,340	>20,000
			163	268	>2,000
12	2	*dublin*	117,000	1,488,000	7,200,000
			3,200	>17,350	>200,000
			32	74	470

Table V. *Survival of* S. typhi *in different types of butter*

Period of storage (days)	Stored at 15° C. Type of butter				Stored at 3° C. Type of butter			
	English	Foreign	Empire salted	Empire unsalted	English	Foreign	Empire salted	Empire unsalted
—	+	+	+	+	+	+	+	+
80	+	+	+	+	+	+	+	+
108	+	+	–	–	+	+	+	+
126	C	C	C	C	–	–	–	–

+ = survival demonstrated by culture of butter.
– = no growth in cultures.
C = complete contamination of culture.

When fresh unsalted butter was contaminated with other members of the genus (Table VI), all survived after 38 days' storage, but by the 68th day *S. cholerae-suis* had died out in the sample stored at 15° C. This organism was still demonstrable in the refrigerated sample on the 90th but had dis-

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Table VI. *Survival of various species of* Salmonella *in English butter*

Period of storage (days)	Survival of *Salmonella* in English butter											
	dublin		*cholerae-suis*		*paratyphi* B		*typhi-murium*		*enteritidis*		*bovis-morbificans*	
	15°	3°	15°	3°	15°	3°	15°	3°	15°	3°	15°	3°
—	+	+	+	+	+	+	+	+	+	+	+	+
38	+	+	+	+	+	+	+	+	+	+	+	+
68	+	+	−	+	+	+	+	+	+	+	+	+
90	+	+	−	+	+	+	+	+	+	+	+	+
98	+	+	−	−	+	+	+	+	+	+	+	+
112	C	+	C	−	C	+	C	+	C	+	C	+

appeared by the 98th day. All other species of *Salmonella* examined were still present on the 98th day in samples stored at both 15 and 3° C. After 112 days the cultures made from butter samples stored at 15° C. were completely overgrown by contaminants whilst the *Salmonella* organisms were still viable in those stored at 3° C.

General discussion

The experimental results which have been recorded show clearly that those *Salmonella* which are likely to contaminate milk supplies and which cause either enteric fever or food-poisoning in man, grow readily in freshly drawn milk stored under normal commercial or household conditions when refrigeration is not resorted to. Obviously, therefore, any effect exerted by the growth-inhibitory factor of fresh milk is very transient, and since multiplication was observed even after small initial inocula had been added to the milk, it would appear that the inhibitory factor was not bactericidal for *Salmonella*. While the results of the experiment suggest that most rapid multiplication occurs after moderate contamination of the milk, it is doubtful if this point is of real significance. In any case, it seems evident that, whether the contamination originates from the cow, as usually happens with *S. dublin*, or from some external source as with *S. typhi*, the really important factor in the epidemiology of milk-borne *Salmonella* outbreaks is that the contaminating organism can thrive during storage and thus cause wholesale contamination of pooled milk. A point of some interest which has arisen out of the investigation is the fact that strains of *S. typhi* recently isolated from human cases multiplied more rapidly in milk than did old laboratory stock cultures.

When artificially incorporated in butter after manufacture, these *Salmonella* were found to survive for periods varying from 2 to 3 months and even longer when the butter was stored at 15 or 3° C. Neither the quality of the butter nor the presence or absence of salt appeared to influence this survival. There was no suggestion that any multiplication occurred, and after about 2 months' storage the organisms began to decrease in numbers. The dying-off of organisms occurred in the first place near the surface of the butter, possibly owing to the result of oxidation processes.

SUMMARY AND CONCLUSIONS

1. A technique for studying the behaviour of *Salmonella* in milk is described.

2. *S. typhi*, *paratyphi* B, *dublin*, *cholerae-suis*, *typhi-murium*, *enteritidis* and *bovis-morbificans* all multiplied readily even in absolutely fresh raw milk stored at 15° C., and in commercial grades of raw and of heat-treated milk rapid multiplication also occurred.

3. Strains of *S. typhi* recently isolated from man showed more rapid multiplication in milk than did stock laboratory cultures.

4. These *Salmonella* all survived at least 2 months when incorporated in various grades of salted and unsalted butter.

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REFERENCES

ANDRESEN, P. H. (1932). *Z. Hyg. InfektKr.* **113**, 530.
BRUCK, C. (1903). *Dtsch. Med. Wschr.* **29**, 460.
CAUTLEY, E. (1896–97). *Rep. Med. Off. Loc. Govt. Board, London.*
EYRE, J. W. (1904). *J. State Med.* **12**, 728.
KAISER (1921). *Zlb. Bakt.* Orig. **86**, 554.
PFUHL (1902). *Z. Hyg. InfektKr.* **40**, 555.
PULLINGER, E. J. & KEMP, AUDREY E. (1937). *J. Hyg., Camb.*, **37**, 527.
WADE, E. M., FELLOW, A. P. H. & SHERE, L. (1928). *Amer. J. publ. Hlth*, **18**, 1480.

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