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Update on Preenrichment and Selective Enrichment Conditions for Detection of *Salmonella* in Foods

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(Received for publication June 30, 1980)

ABSTRACT

The search for short and sensitive cultural methods for detection of *Salmonella* in foods has met with limited success. Short (3-8 h) incubation of non-selective enrichment media do not provide conditions for effective resuscitation of stressed or injured salmonellae and result in unacceptably high numbers of false-negative results. Isolation of *Salmonella* is not dependent on the nutritional value of preenrichment media; simple media such as lactose and nutrient broths are equally reliable as highly nutritive sterility testing media. The need for detergents in non-selective enrichment of fatty foods and use of preenrichment transfer volumes greater than 1 ml is not indicated. Although selective enrichment in tetrathionate brilliant green (TBG) broth at an elevated temperature (43 C) increases method sensitivity, use of Mueller-Kauffman TBG under similar analytical conditions may be inhibitory to *Salmonella*. Refrigeration of preenrichment and selective enrichment broth cultures has been used successfully to provide greater analytical flexibility by increasing the number of days on which analyses can be initiated without engendering weekend work.

Standard cultural procedures for isolation of *Salmonella* in foods are time-consuming and require a minimum of 4-5 days to obtain presumptive evidence of *Salmonella* contamination (1,26,28,51). These procedures generally include preenrichment of food samples in a non-selective broth medium, enrichment in a selective broth medium, isolation of presumptive colonies on differential plating media, biochemical screening and serological confirmation of presumptive isolates. Preenrichment in a non-selective broth medium provides for the non-inhibited growth of indigenous bacterial flora and the resuscitation and proliferation of stressed or injured salmonellae to detectable levels (35). Selective enrichment inhibits growth of non-salmonellae and facilitates isolation of *Salmonella* on differential plating media (17). Selective agar media commonly used for presumptive identification of *Salmonella* respond to lactose and/or sucrose utilization except bismuth sulfite agar, which reacts to bacterial production of hydrogen sulfide (7). The increasing incidence of *Salmonella* biotypes that ferment lactose (3,18) or show urease activity (19) underlines the growing limitations of biochemical profiles as a reliable taxonomic tool, and the

importance of serological confirmation of isolates with somatic and flagellar antisera.

This paper reviews the significance of different preenrichment and selective enrichment conditions in the isolation of *Salmonella* from foods.

NON-SELECTIVE ENRICHMENT [PREENRICHMENT]

Several preenrichment media of varying nutritive value are used in standard cultural procedures (Table 1). Although lactose broth and buffered peptone water (BPW) are preferred by U.S. and international agencies, respectively, agreement can be found in the use of brilliant green water for preenrichment of nonfat dried milk and dried whole milk and of reconstituted nonfat dried milk with added brilliant green for chocolate and other confectionery products. Lack of unanimity in the choice of a non-selective enrichment medium apparently stems from few detailed studies on productivity of preenrichment media under different experimental conditions. The performance of Clausen, EE (Mossel), Eugon, GN, lactose, nutrient, and tergitol-7 broths

TABLE 1. Media for non-selective enrichment.

Agency/Group	Medium
AOAC (2) and APHA (1)	Lactose Brilliant green water
FDA (51)	Lactose Tryptic soy Brilliant green water Powdered milk with brilliant green Nutrient
HPB (26)	Nutrient Brilliant green water Trypticase soy Powdered milk with brilliant green
ICMSF (28)	Lactose Buffered peptone water Brilliant green water Powdered milk with brilliant green
ISO (29,30)	Buffered peptone water Ringer's with brilliant green
NAS (42)	Lactose Brilliant green water
USDA (50)	Lactose

(Table 2) was recently compared and, except for EE broth, found to be equally effective for isolation of *Salmonella* in naturally contaminated low and high moisture foods (9, and Table 3). These findings indicate that the nutrient level of preenrichment media is not a determinant for effective isolation of *Salmonella* from foods. Similar recoveries have been reported with BPW and lactose broth enrichment of egg products (55), BPW and tryptone soya broth enrichment of minced meat (52), and BPW, reconstituted nonfat dried milk with added brilliant green, trypticase soy, and lactose broth enrichment of milk chocolate (60). Although similar in productivity, lactose and nutrient broths identified fewer naturally contaminated gelatin samples than tryptic soy broth (43).

Short preenrichment incubation periods have been evaluated for their ability to abbreviate standard cultural procedures without adversely affecting method sensitivity. Data supporting use of short incubation periods have been obtained with *Salmonella*-infected foods and animal feeds preenriched with continuous shaking for 7 h in a modified M broth (27). Good recovery of heat-injured salmonellae was also reported for milk powder preenriched for 2-6 h in BPW (54). Preenrichment of dry foods for 6 h in shallow layers of tryptone soya peptone broth was superior to overnight incubation in lactose broth for recovery of *Enterobacteriaceae* (40). Evidence of the limited reliability of short incubation periods is equally of interest. Studies on isolation of injured salmonellae from foods showed that resuscitation for up to 6 h in lactose or tryptone soya broths was insufficient for repair and active growth of the organisms (53). Preenrichment for 24 h rather than 7 h in FAS broth (Difco) markedly increased the immunofluorescent detection of *Salmonella* in food and animal feeds (37). Recent work on the efficacy of seven preenrichment culture media showed that short (6 h) incubation periods are unreliable and produce unacceptably high numbers of false-negative results (9, and Table 3). Similar results were obtained with fish and shellfish preenriched for 3 h

TABLE 3. Recovery of *Salmonella* with different preenrichment media.^{1,2}

Medium	Salmonella-positive samples	
	Incubation time	
	6 h	24 h
Clausen	15	23
EE (Mossel)	8	17
Eugon	15	22
GN	14	22
Lactose	15	23
Nutrient	14	23
Tergitol-7	11	23

¹A total of 23 positive samples examined.

²Replicate food samples (100 g) were preenriched in 900 ml of each culture medium; preenrichment cultures were selectively enriched in TBG₄₃ and SC₃₅ and plated on BiS and BGS agar media.

in lactose broth (10).

Standard methods generally recommend use of 0.5-1.0% anionic Tergitol-7 (sodium heptadecyl sulfate) or other suitable detergents for effective recovery of *Salmonella* in fatty foods (Table 4). Presumably surfactants liberate salmonellae trapped in lipid micelles into the culture medium where the organisms grow to detectable levels. Although Tergitol-7 increased the recovery of *Salmonella* from pork sausages (21,38), other studies have found this agent to be of little or no diagnostic value and to inhibit *Salmonella* and other *Enterobacteriaceae* (12,39,45). Preliminary results (D'Aoust, unpublished) on recovery of *Salmonella* from naturally contaminated fatty foods preenriched in nutrient broth supplemented with 3-10% (w/v) surfactant fail to support use of these agents to increase method sensitivity (Table 5). Each contaminated food was detected under all analytical conditions except for a single false-negative result with Myrj 52S; salmonellae populations in supplemented and control preenrichment cultures ranged from 10⁰-10⁸ cells/ml with median counts of 10⁴-10⁵ cells/ml.

TABLE 2. Composition of selected preenrichment media.

Broth medium	Ingredients		Recommended for
	Extract	Saccharide	
Clausen	Tryptone Peptone Yeast extract	Dextrose Glycerol	Sterility testing
EE (Mossel)	Tryptose	Dextrose	<i>Enterobacteriaceae</i>
Eugon	Peptone	Dextrose	Sterility testing
GN	Peptone	Dextrose Mannitol	<i>Salmonella-Shigella</i>
Nutrient	Peptone Beef extract	None	General purpose
Lactose	Peptone Beef extract	Lactose	Coliforms
Tergitol-7	Peptone Yeast extract	Lactose	<i>Salmonella</i> and enteric bacilli

TABLE 4. *Detergents in non-selective enrichment.*

Agency/Group	Detergent	Concentration (%)
FDA (51)	Tergitol-7	1.0
	Triton X-100	To initiate foaming
HPB (26)	Tergitol-7	0.6
ICMSF (28)	Tergitol-7	0.2-1.0
ISO (29)	Tergitol-7	1.0
NAS (42)	Tergitol-7	0.6
USDA (50)	Tergitol-7	0.5-1.0

TABLE 5. *Detergents for recovery of Salmonella in fatty foods.*^{1,2}

Detergent ²	Number of positive samples	Log ₁₀ <i>Salmonella</i> /ml preenrichment culture		
		Low	High	Median
Arlacel 80 + Tween 60	20	0	7	4
MYRJ 52S	19	0	8	4
Tergitol-7	20	3	7	5
Triton X-100	20	2	8	5
Tween 80	20	1	7	4
Nutrient broth control	20	0	7	4

¹A total of 20 naturally contaminated foods analyzed.

²Replicate food samples (100 g) were preenriched in nutrient broth containing 3% (w/v) detergent except Arlacel 80 + Tween 60 where 10% (w/v) was used. Preenrichment cultures were selectively enriched in TBG₄₃ and SC₃₅ and plated on BiS and BGS agar media.

The amount of preenrichment culture transferred into selective enrichment medium (transfer volume) differs with standard methods (Table 6). In a recent study on the efficacy of different preenrichment conditions, transfer volume-dependent recovery was observed only with short (6 h) preenrichment incubation periods; at 24 h of incubation, 10 ml and 1 ml transfer volumes were equally sensitive (Table 7). No significant differences in recoveries were reported upon examination of the effect of 1-ml and 2-ml transfer volumes on isolation of salmonellae in artificially contaminated dry foods (44). Limited data also indicate that 1-ml transfer volumes can readily recover *Salmonella* from 1500-g samples of dry foods preenriched in 13.5 litres of broth (48). Clearly, transfer volumes greater than 1 ml do not increase method sensitivity and substantially increase analytical costs through use of greater volumes of selective enrichment media.

SELECTIVE ENRICHMENT

Direct enrichment (no preenrichment) of clinical specimens in selective broth media is not uncommon, and the approach has been adopted for raw or highly contaminated food products (1,51). It is important to note that the bacterial characteristics of these two types of samples differ markedly; in clinical samples, large populations of physiologically active organisms facilitate detection, whereas comparatively few, inactive salmonellae may contaminate food samples. Detection of

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TABLE 6. *Preenrichment transfer volumes.*

Agency/Group	Sample (g)/culture volume	Transfer volume (ml)
AOAC (2) and APHA (1)	25/225	1.0
FDA (51)	25/225	1.0
HPB (26)	25/225	1.0
ICMSF (28)	25/225	1.0
ISO (29)	25/225	10.0
NAS (42)	25/225	1.0
USDA (50)	25/225	0.5
	Large composite	10.0

TABLE 7. *Effect of preenrichment transfer volume on recovery.*^{1,2}

Medium	Incubation time (h)	Salmonella-positive samples		
		Transfer volume (ml)		
		10	1.0	0.1
Clausen	6	19	15	7
	24	23	23	23
EE (Mossel)	6	10	8	4
	24	17	17	17
Eugon	6	19	15	9
	24	22	22	22
GN	6	19	14	5
	24	22	22	22
Lactose	6	20	15	8
	24	23	23	22
Nutrient	6	21	14	5
	24	23	23	23
Tergitol-7	6	18	11	5
	24	23	23	22
TOTAL:	6	126	92	43
	24	153	153	151

¹A total of 23 positive samples examined.

²See Table 3.

Salmonella in raw meats and sewage materials was substantially greater with preenrichment than with direct enrichment (Table 8). Direct enrichment of food samples heavily contaminated with competitive flora is not justified and may lead to false-negative results.

The multiplicity of time-temperature regimens and selective enrichment broths used in standard cultural procedures is notable (Table 9). In addition to the tetrathionate and selenite cystine (SC) broths, selenite brilliant green (4,5,49) and Rappaport (57,59) enrichment media have also been used in food analysis. Several formulations of tetrathionate broths, which differ widely in composition and selectivity, are currently in use (Table 10). These include TBG, the Mueller (41) tetrathionate broth as modified by Kauffman (33); TT, the formulation of Hajna (23); Tet, as formulated by Rolfe (47); MK TBG, the Mueller-Kauffman tetrathionate broth widely used in Europe (14,24,31,58) and marketed by Oxoid Limited. The tetrathionate (S₄O₆⁼)

TABLE 8. *Efficacy of preenrichment and direct enrichment.*

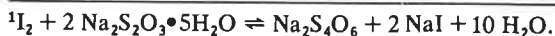
Sample material	Preenrichment/ enrichment	Percent recovery by		
		Preenrichment	Direct enrichment	Reference
Minced meat (artificially)	BPW/MK TBG ₄₃	90	27	Edel (15)
Minced meat (naturally)	BPW/MK TBG ₄₃	83	56	
Meat	LAC/TBG ₃₅	91	43	Gabis (20)
	LAC/SC ₃₅	91	62	
Sewage material	BPW/MK TBG ₄₃	100	55	Grunnett (22)
Sewage water	BPW/MK TBG ₄₃	100	51	Harvey (24)

TABLE 9. *Selective enrichment conditions in standard methods.*

Agency/Group	Media	Incubation time (h)
AOAC (2) and APHA (1)	TBG ₃₅ , SC ₃₅	24 ± 2
FDA (51)	TBG ₃₅ , SC ₃₅	24 ± 2
HPB (26)	TBG ₄₃ , SC ₃₅	24 ± 2
ICMSF (28)	TBG ₄₃ , SC ₄₃	24
ISO (29)	MK TBG ₄₃ , SC ₃₅	24 and 48
NAS (42)	TBG ₃₅ , SC ₃₅	18-24
USDA (50)	TT ₃₅	18-24

TABLE 10. *Characteristics of tetrathionate¹ enrichment broths.*

Characteristic	TBG	TT (Hajna)	TET (Rolfe)	MK TBG
I ₂ (g/l)	6.0	5.0	11	4.0
KI (g/l)	5.0	8.0	13.75	5.0
Molarity I ₂ = S ₄ O ₆ ⁼	0.024	0.020	0.043	0.016
Excess S ₂ O ₃ ⁼ (moles)	0.072	0.11	NONE	0.17
Brilliant green (g/l)	0.01	0.01	0.01	0.01
Bile salts (g/l)	1.0	0.5	NONE	5.0
CaCO ₃ (g/l)	10.	25.0	50.0	45.0



anion constitutes the principal selective agent in these enrichment media and is most effective at a concentration of 0.03 M (34). The high S₄O₆⁼ content and absence of bile salts to neutralize the toxicity of brilliant green (36) in the Rolfe formulation underlines the probable toxicity of this medium to salmonellae. Differences in CaCO₃ content (Table 10) further indicate the limited ability of some formulations to neutralize tetrathionate reductase-linked acid production (34). The excess of S₂O₃⁼ which may be inhibitory to salmonellae (34), and the greater bile salts content of the MK TBG formulation may account, in part, for the reported toxicity of this medium with pure *Salmonella* cultures and raw meat samples (Table 11).

Incubation temperature markedly affects productivity of selective enrichment broths by suppressing growth of competing microorganisms but not *Salmonella*. Since the first report of increased recovery of salmonellae from clinical specimens using selenite broth incubated at 43 C (25), several studies involving different analytical schemes and sample material have shown the net

TABLE 11. *Toxicity of the MK tetrathionate enrichment medium.*

Sample material	Percent recovery		Reference
	35-37 C	43 C	
Heat-injured cultures ²	90	< 1	Corry (6)
Pure cultures ²	60	30	Vassiliadis (58)
Pork sausage ²	ND ³	6	Roberts (46)
Meat filtrate ²	36	44	Kafel (31)
Pork sausage ¹	ND ³	64	Vassiliadis (57)
Pure cultures ²	86	72	van Schothorst (56)

¹With non-selective preenrichment.

²Direct enrichment.

³ND = Not done.

advantage of incubation at elevated temperatures (Table 12). Although international organizations such as the I.C.M.S.F. and ISO recommend high-temperature incubation of enrichment broths, federal agencies and health associations in the United States have not yet opted for this approach in their analytical procedures (Table 10).

Standard methodology recommends incubation of selective enrichment broths for 18-24 h (Table 9); however, incubation periods greater than 24 h are used (29,30) and, on occasion, have been found to yield greater numbers of *Salmonella*-positive samples irrespective of enrichment conditions (Table 13). Claims of increased recovery with prolonged incubation originate mainly from European laboratories using the inhibitory MK

TABLE 12. *Efficacy of selective enrichment at elevated temperatures*

Sample material	Enrichment	Percent recovery		Reference
		35-37 C	43 C	
Animal feed	SBGS ²	58	84	Carlson (5)
Feces	TET (Rolfe) ²	66	75	Edel (13)
	Selenite F ²	64	74	
	TET (Oxoid) ²	74	98	
Pork sausage	TBG ²	43	57	Morris (38)
Red meat	SC ¹	76	92	Silliker (49)
	SBGS ¹	82	91	
	TET ¹	64	81	
	TBG ¹	64	86	
Sewage water	MK TBG ²	38	96	Grunnett (22)

¹With non-selective enrichment.

²Direct enrichment.

TBG at 43 C. Some of our preliminary work with food samples preenriched in nutrient broth suggests that such increased recovery is not dependent on *Salmonella* growth (8). *Salmonella* counts in TBG₄₃ and SC₃₅ broths incubated for 16-48 h ranged between 10⁵-10⁶ cells/ml after 16 h of incubation with a one log maximum reduction in counts after 48 h. Increased recovery of salmonellae with prolonged incubation presumably results from differential death rate kinetics; competitive flora, because of their greater sensitivity to elevated temperatures and toxicity of selective enrichment media, die off more rapidly, thus facilitating recovery of *Salmonella* on selective plating media.

The need for increased laboratory efficiency and flexibility in analytical procedures led us to consider refrigeration of preenrichment and enrichment broth cultures for 72 h as a means of increasing the number of days on which sample analyses could be initiated without engendering weekend work. Presently, preenrichment of food samples is restricted to Mondays and Tuesdays to obtain, the following Friday, presumptive evidence of *Salmonella* on selective agar media. With the refrigeration approach, enrichment and preenrichment broth cultures of samples analyzed on Wednesday and Thursday, respectively, would be refrigerated on Friday for the weekend and sample analysis resumed the following Monday. A recent evaluation of this novel approach showed that recovery of *Salmonella* from low and high moisture foods under standard and refrigerated conditions were identical, except for a single contaminated pork liver sample which could not be identified with the refrigerated preenrichment culture (Table 14). Six laboratories participated in the analysis of poultry samples; two of these laboratories contributed 19 of the 21 false-negative results obtained with this food product

TABLE 14. Recovery from refrigerated preenrichment and enrichment broth cultures.¹

Food	Salmonella-positive samples		
	Standard cultural	Refrigerated preenrichment	Refrigerated enrichment
Poultry	105	93 (90)	96 (91)
High moisture	21	20 (95)	21 (100)
Low moisture	34	34 (100)	34 (100)
Total	160 (100)	147 (92)	151 (94)

¹Percent values are in brackets.

(11). The encouraging results obtained with this analytical scheme, and the need for greater method flexibility has led to an international I.C.M.S.F. evaluation of the refrigeration approach.

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TABLE 13. Recovery with prolonged incubation of selective enrichment media.

	Sample material	Preenrichment/enrichment	Percent recovery after			
			24 h	48 h	72 h	96 h
<i>Preenrichment</i>						
Edel (15)	Minced meat (artificially)	BPW/MK TBG ₄₃	90	95		
	Minced meat (naturally)	BPW/MK TBG ₄₃	83	92		
Edel (16)	Animal feed	BPW/MK TBG ₄₃	95	100		
Harvey (24)	Sewage water	BPW/MK TBG ₄₃	64	100		
Vassiliadis (57)	Pork sausage	BPW/MK TBG ₄₃	52	64		
<i>Direct enrichment</i>						
Carlson (5)	Animal feed	NA ¹ /SBGS ₃₇	58	53		58
		NA/SBGS ₄₃	84	87		82
Kampelmacher (32)	Minced meat	NA/MK TBG ₄₃	75	100		
Edel (14)	Minced meat (artificially)	NA/MK TBG ₄₃	99	100		
	Feces (artificially)	NA/MK TBG ₄₃	80	99		
Edel (15)	Minced meat (artificially)	NA/MK TBG ₄₃	27	52		
	Minced meat (naturally)	NA/MK TBG ₄₃	56	69		
Grunnett (22)	Sewage water	NA/MK TBG ₄₃	60	80	95	100
Kafel (31)	Meat filtrate	NA/MK TBG ₃₇	36	34		
		NA/MK TBG ₄₃	44	59		

¹NA = Not applicable.

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Exhibit 1054
Mullins Decl. Appendix J-2
(D'Aoust from Science Direct)

Update on Preenrichment and Selective Enrichment Conditions for Detection of *Salmonella* in Foods

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ABSTRACT

The search for short and sensitive cultural methods for detection of *Salmonella* in foods has met with limited success. Short (3-8 h) incubation of non-selective enrichment media do not provide conditions for effective resuscitation of stressed or injured salmonellae and result in unacceptably high numbers of false-negative results. Isolation of *Salmonella* is not dependent on the nutritional value of preenrichment media; simple media such as lactose and nutrient broths are equally reliable as highly nutritive sterility testing media. The need for detergents in non-selective enrichment of fatty foods and use of preenrichment transfer volumes greater than 1 ml is not indicated. Although selective enrichment in tetrathionate brilliant green (TBG) broth at an elevated temperature (43 C) increases method sensitivity, use of Mueller-Kauffman TBG under similar analytical conditions may be inhibitory to *Salmonella*. Refrigeration of preenrichment and selective enrichment broth cultures has been used successfully to provide greater analytical flexibility by increasing the number of days on which analyses can be initiated without engendering weekend work.

Standard cultural procedures for isolation of *Salmonella* in foods are time-consuming and require a minimum of 4-5 days to obtain presumptive evidence of *Salmonella* contamination (1,26,28,51). These procedures generally include preenrichment of food samples in a non-selective broth medium, enrichment in a selective broth medium, isolation of presumptive colonies on differential plating media, biochemical screening and serological confirmation of presumptive isolates. Preenrichment in a non-selective broth medium provides for the non-inhibited growth of indigenous bacterial flora and the resuscitation and proliferation of stressed or injured salmonellae to detectable levels (35). Selective enrichment inhibits growth of non-salmonellae and facilitates isolation of *Salmonella* on differential plating media (17). Selective agar media commonly used for presumptive identification of *Salmonella* respond to lactose and/or sucrose utilization except bismuth sulfite agar, which reacts to bacterial production of hydrogen sulfide (7). The increasing incidence of *Salmonella* biotypes that ferment lactose (3,18) or show urease activity (19) underlines the growing limitations of biochemical profiles as a reliable taxonomic tool, and the

importance of serological confirmation of isolates with somatic and flagellar antisera.

This paper reviews the significance of different preenrichment and selective enrichment conditions in the isolation of *Salmonella* from foods.

NON-SELECTIVE ENRICHMENT [PREENRICHMENT]

Several preenrichment media of varying nutritive value are used in standard cultural procedures (Table 1). Although lactose broth and buffered peptone water (BPW) are preferred by U.S. and international agencies, respectively, agreement can be found in the use of brilliant green water for preenrichment of nonfat dried milk and dried whole milk and of reconstituted nonfat dried milk with added brilliant green for chocolate and other confectionery products. Lack of unanimity in the choice of a non-selective enrichment medium apparently stems from few detailed studies on productivity of preenrichment media under different experimental conditions. The performance of Clausen, EE (Mossel), Eugon, GN, lactose, nutrient, and tergitol-7 broths

TABLE 1. Media for non-selective enrichment.

Agency/Group	Medium
AOAC (2) and APHA (1)	Lactose Brilliant green water
FDA (51)	Lactose Tryptic soy Brilliant green water Powdered milk with brilliant green Nutrient
HPB (26)	Nutrient Brilliant green water Trypticase soy Powdered milk with brilliant green
ICMSF (28)	Lactose Buffered peptone water Brilliant green water Powdered milk with brilliant green
ISO (29,30)	Buffered peptone water Ringer's with brilliant green
NAS (42)	Lactose Brilliant green water
USDA (50)	Lactose

(Table 2) was recently compared and, except for EE broth, found to be equally effective for isolation of *Salmonella* in naturally contaminated low and high moisture foods (9, and Table 3). These findings indicate that the nutrient level of preenrichment media is not a determinant for effective isolation of *Salmonella* from foods. Similar recoveries have been reported with BPW and lactose broth enrichment of egg products (55), BPW and tryptone soya broth enrichment of minced meat (52), and BPW, reconstituted nonfat dried milk with added brilliant green, trypticase soy, and lactose broth enrichment of milk chocolate (60). Although similar in productivity, lactose and nutrient broths identified fewer naturally contaminated gelatin samples than tryptic soy broth (43).

Short preenrichment incubation periods have been evaluated for their ability to abbreviate standard cultural procedures without adversely affecting method sensitivity. Data supporting use of short incubation periods have been obtained with *Salmonella*-infected foods and animal feeds preenriched with continuous shaking for 7 h in a modified M broth (27). Good recovery of heat-injured salmonellae was also reported for milk powder preenriched for 2-6 h in BPW (54). Preenrichment of dry foods for 6 h in shallow layers of tryptone soya peptone broth was superior to overnight incubation in lactose broth for recovery of *Enterobacteriaceae* (40). Evidence of the limited reliability of short incubation periods is equally of interest. Studies on isolation of injured salmonellae from foods showed that resuscitation for up to 6 h in lactose or tryptone soya broths was insufficient for repair and active growth of the organisms (53). Preenrichment for 24 h rather than 7 h in FAS broth (Difco) markedly increased the immunofluorescent detection of *Salmonella* in food and animal feeds (37). Recent work on the efficacy of seven preenrichment culture media showed that short (6 h) incubation periods are unreliable and produce unacceptably high numbers of false-negative results (9, and Table 3). Similar results were obtained with fish and shellfish preenriched for 3 h

TABLE 3. Recovery of *Salmonella* with different preenrichment media.^{1,2}

Medium	Salmonella-positive samples Incubation time	
	6 h	24 h
Clausen	15	23
EE (Mossel)	8	17
Eugon	15	22
GN	14	22
Lactose	15	23
Nutrient	14	23
Tergitol-7	11	23

¹A total of 23 positive samples examined.

²Replicate food samples (100 g) were preenriched in 900 ml of each culture medium; preenrichment cultures were selectively enriched in TBG₄₃ and SC₃₅ and plated on BiS and BGS agar media.

in lactose broth (10).

Standard methods generally recommend use of 0.5-1.0% anionic Tergitol-7 (sodium heptadecyl sulfate) or other suitable detergents for effective recovery of *Salmonella* in fatty foods (Table 4). Presumably surfactants liberate salmonellae trapped in lipid micelles into the culture medium where the organisms grow to detectable levels. Although Tergitol-7 increased the recovery of *Salmonella* from pork sausages (21,38), other studies have found this agent to be of little or no diagnostic value and to inhibit *Salmonella* and other *Enterobacteriaceae* (12,39,45). Preliminary results (D'Aoust, unpublished) on recovery of *Salmonella* from naturally contaminated fatty foods preenriched in nutrient broth supplemented with 3-10% (w/v) surfactant fail to support use of these agents to increase method sensitivity (Table 5). Each contaminated food was detected under all analytical conditions except for a single false-negative result with Myrj 52S; salmonellae populations in supplemented and control preenrichment cultures ranged from 10⁰-10⁸ cells/ml with median counts of 10⁴-10⁵ cells/ml.

TABLE 2. Composition of selected preenrichment media.

Broth medium	Ingredients		Recommended for
	Extract	Saccharide	
Clausen	Tryptone Peptone Yeast extract	Dextrose Glycerol	Sterility testing
EE (Mossel)	Tryptose	Dextrose	<i>Enterobacteriaceae</i>
Eugon	Peptone	Dextrose	Sterility testing
GN	Peptone	Dextrose Mannitol	<i>Salmonella-Shigella</i>
Nutrient	Peptone Beef extract	None	General purpose
Lactose	Peptone Beef extract	Lactose	Coliforms
Tergitol-7	Peptone Yeast extract	Lactose	<i>Salmonella</i> and enteric bacilli

TABLE 4. *Detergents in non-selective enrichment.*

Agency/Group	Detergent	Concentration (%)
FDA (51)	Tergitol-7	1.0
	Triton X-100	To initiate foaming
HPB (26)	Tergitol-7	0.6
ICMSF (28)	Tergitol-7	0.2-1.0
ISO (29)	Tergitol-7	1.0
NAS (42)	Tergitol-7	0.6
USDA (50)	Tergitol-7	0.5-1.0

TABLE 5. *Detergents for recovery of Salmonella in fatty foods.*^{1,2}

Detergent ²	Number of positive samples	Log ₁₀ <i>Salmonella</i> /ml preenrichment culture		
		Low	High	Median
Arlacel 80 + Tween 60	20	0	7	4
MYRJ 52S	19	0	8	4
Tergitol-7	20	3	7	5
Triton X-100	20	2	8	5
Tween 80	20	1	7	4
Nutrient broth control	20	0	7	4

¹A total of 20 naturally contaminated foods analyzed.

²Replicate food samples (100 g) were preenriched in nutrient broth containing 3% (w/v) detergent except Arlacel 80 + Tween 60 where 10% (w/v) was used. Preenrichment cultures were selectively enriched in TBG₄₃ and SC₃₅ and plated on BiS and BGS agar media.

The amount of preenrichment culture transferred into selective enrichment medium (transfer volume) differs with standard methods (Table 6). In a recent study on the efficacy of different preenrichment conditions, transfer volume-dependent recovery was observed only with short (6 h) preenrichment incubation periods; at 24 h of incubation, 10 ml and 1 ml transfer volumes were equally sensitive (Table 7). No significant differences in recoveries were reported upon examination of the effect of 1-ml and 2-ml transfer volumes on isolation of salmonellae in artificially contaminated dry foods (44). Limited data also indicate that 1-ml transfer volumes can readily recover *Salmonella* from 1500-g samples of dry foods preenriched in 13.5 litres of broth (48). Clearly, transfer volumes greater than 1 ml do not increase method sensitivity and substantially increase analytical costs through use of greater volumes of selective enrichment media.

SELECTIVE ENRICHMENT

Direct enrichment (no preenrichment) of clinical specimens in selective broth media is not uncommon, and the approach has been adopted for raw or highly contaminated food products (1,51). It is important to note that the bacterial characteristics of these two types of samples differ markedly; in clinical samples, large populations of physiologically active organisms facilitate detection, whereas comparatively few, inactive salmonellae may contaminate food samples. Detection of

TABLE 6. *Preenrichment transfer volumes.*

Agency/Group	Sample (g)/culture volume	Transfer volume (ml)
AOAC (2) and APHA (1)	25/225	1.0
FDA (51)	25/225	1.0
HPB (26)	25/225	1.0
ICMSF (28)	25/225	1.0
ISO (29)	25/225	10.0
NAS (42)	25/225	1.0
USDA (50)	25/225	0.5
	Large composite	10.0

TABLE 7. *Effect of preenrichment transfer volume on recovery.*^{1,2}

Medium	Incubation time (h)	Salmonella-positive samples		
		Transfer volume (ml)		
		10	1.0	0.1
Clausen	6	19	15	7
	24	23	23	23
EE (Mossel)	6	10	8	4
	24	17	17	17
Eugon	6	19	15	9
	24	22	22	22
GN	6	19	14	5
	24	22	22	22
Lactose	6	20	15	8
	24	23	23	22
Nutrient	6	21	14	5
	24	23	23	23
Tergitol-7	6	18	11	5
	24	23	23	22
TOTAL:	6	126	92	43
	24	153	153	151

¹A total of 23 positive samples examined.

²See Table 3.

Salmonella in raw meats and sewage materials was substantially greater with preenrichment than with direct enrichment (Table 8). Direct enrichment of food samples heavily contaminated with competitive flora is not justified and may lead to false-negative results.

The multiplicity of time-temperature regimens and selective enrichment broths used in standard cultural procedures is notable (Table 9). In addition to the tetrathionate and selenite cystine (SC) broths, selenite brilliant green (4,5,49) and Rappaport (57,59) enrichment media have also been used in food analysis. Several formulations of tetrathionate broths, which differ widely in composition and selectivity, are currently in use (Table 10). These include TBG, the Mueller (41) tetrathionate broth as modified by Kauffman (33); TT, the formulation of Hajna (23); Tet, as formulated by Rolfe (47); MK TBG, the Mueller-Kauffman tetrathionate broth widely used in Europe (14,24,31,58) and marketed by Oxoid Limited. The tetrathionate (S₄O₆⁼)

TABLE 8. *Efficacy of preenrichment and direct enrichment.*

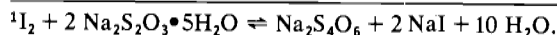
Sample material	Preenrichment/ enrichment	Percent recovery by		
		Preenrichment	Direct enrichment	Reference
Minced meat (artificially)	BPW/MK TBG ₄₃	90	27	Edel (15)
Minced meat (naturally)	BPW/MK TBG ₄₃	83	56	
Meat	LAC/TBG ₃₅	91	43	Gabis (20)
	LAC/SC ₃₅	91	62	
Sewage material	BPW/MK TBG ₄₃	100	55	Grunnett (22)
Sewage water	BPW/MK TBG ₄₃	100	51	Harvey (24)

TABLE 9. *Selective enrichment conditions in standard methods.*

Agency/Group	Media	Incubation time (h)
AOAC (2) and		
APHA (1)	TBG ₃₅ , SC ₃₅	24 ± 2
FDA (51)	TBG ₃₅ , SC ₃₅	24 ± 2
HPB (26)	TBG ₄₃ , SC ₃₅	24 ± 2
ICMSF (28)	TBG ₄₃ , SC ₄₃	24
ISO (29)	MK TBG ₄₃ , SC ₃₅	24 and 48
NAS (42)	TBG ₃₅ , SC ₃₅	18-24
USDA (50)	TT ₃₅	18-24

TABLE 10. *Characteristics of tetrathionate¹ enrichment broths.*

Characteristic	TBG	TT (Hajna)	TET (Rolfe)	MK TBG
I ₂ (g/l)	6.0	5.0	11	4.0
KI (g/l)	5.0	8.0	13.75	5.0
Molarity I ₂ = S ₄ O ₆ ⁼	0.024	0.020	0.043	0.016
Excess S ₂ O ₃ ⁼ (moles)	0.072	0.11	NONE	0.17
Brilliant green (g/l)	0.01	0.01	0.01	0.01
Bile salts (g/l)	1.0	0.5	NONE	5.0
CaCO ₃ (g/l)	10.	25.0	50.0	45.0



anion constitutes the principal selective agent in these enrichment media and is most effective at a concentration of 0.03 M (34). The high S₄O₆⁼ content and absence of bile salts to neutralize the toxicity of brilliant green (36) in the Rolfe formulation underlines the probable toxicity of this medium to salmonellae. Differences in CaCO₃ content (Table 10) further indicate the limited ability of some formulations to neutralize tetrathionate reductase-linked acid production (34). The excess of S₂O₃⁼ which may be inhibitory to salmonellae (34), and the greater bile salts content of the MK TBG formulation may account, in part, for the reported toxicity of this medium with pure *Salmonella* cultures and raw meat samples (Table 11).

Incubation temperature markedly affects productivity of selective enrichment broths by suppressing growth of competing microorganisms but not *Salmonella*. Since the first report of increased recovery of salmonellae from clinical specimens using selenite broth incubated at 43 C (25), several studies involving different analytical schemes and sample material have shown the net

TABLE 11. *Toxicity of the MK tetrathionate enrichment medium.*

Sample material	Percent recovery		Reference
	35-37 C	43 C	
Heat-injured cultures ²	90	< 1	Corry (6)
Pure cultures ²	60	30	Vassiliadis (58)
Pork sausage ²	ND ³	6	Roberts (46)
Meat filtrate ²	36	44	Kafel (31)
Pork sausage ¹	ND ³	64	Vassiliadis (57)
Pure cultures ²	86	72	van Schothorst (56)

¹With non-selective preenrichment.

²Direct enrichment.

³ND = Not done.

advantage of incubation at elevated temperatures (Table 12). Although international organizations such as the I.C.M.S.F. and ISO recommend high-temperature incubation of enrichment broths, federal agencies and health associations in the United States have not yet opted for this approach in their analytical procedures (Table 10).

Standard methodology recommends incubation of selective enrichment broths for 18-24 h (Table 9); however, incubation periods greater than 24 h are used (29,30) and, on occasion, have been found to yield greater numbers of *Salmonella*-positive samples irrespective of enrichment conditions (Table 13). Claims of increased recovery with prolonged incubation originate mainly from European laboratories using the inhibitory MK

TABLE 12. *Efficacy of selective enrichment at elevated temperatures*

Sample material	Enrichment	Percent recovery		Reference
		35-37 C	43 C	
Animal feed	SBGS ²	58	84	Carlson (5)
Feces	TET (Rolfe) ²	66	75	Edel (13)
	Selenite F ²	64	74	
	TET (Oxoid) ²	74	98	
Pork sausage	TBG ²	43	57	Morris (38)
Red meat	SC ¹	76	92	Silliker (49)
	SBGS ¹	82	91	
	TET ¹	64	81	
	TBG ¹	64	86	
Sewage water	MK TBG ²	38	96	Grunnett (22)

¹With non-selective enrichment.

²Direct enrichment.

TBG at 43 C. Some of our preliminary work with food samples preenriched in nutrient broth suggests that such increased recovery is not dependent on *Salmonella* growth (8). *Salmonella* counts in TBG₄₃ and SC₃₅ broths incubated for 16-48 h ranged between 10⁵-10⁶ cells/ml after 16 h of incubation with a one log maximum reduction in counts after 48 h. Increased recovery of salmonellae with prolonged incubation presumably results from differential death rate kinetics; competitive flora, because of their greater sensitivity to elevated temperatures and toxicity of selective enrichment media, die off more rapidly, thus facilitating recovery of *Salmonella* on selective plating media.

The need for increased laboratory efficiency and flexibility in analytical procedures led us to consider refrigeration of preenrichment and enrichment broth cultures for 72 h as a means of increasing the number of days on which sample analyses could be initiated without engendering weekend work. Presently, preenrichment of food samples is restricted to Mondays and Tuesdays to obtain, the following Friday, presumptive evidence of *Salmonella* on selective agar media. With the refrigeration approach, enrichment and preenrichment broth cultures of samples analyzed on Wednesday and Thursday, respectively, would be refrigerated on Friday for the weekend and sample analysis resumed the following Monday. A recent evaluation of this novel approach showed that recovery of *Salmonella* from low and high moisture foods under standard and refrigerated conditions were identical, except for a single contaminated pork liver sample which could not be identified with the refrigerated preenrichment culture (Table 14). Six laboratories participated in the analysis of poultry samples; two of these laboratories contributed 19 of the 21 false-negative results obtained with this food product

TABLE 14. Recovery from refrigerated preenrichment and enrichment broth cultures.¹

Food	Salmonella-positive samples		
	Standard cultural	Refrigerated preenrichment	Refrigerated enrichment
Poultry	105	93 (90)	96 (91)
High moisture	21	20 (95)	21 (100)
Low moisture	34	34 (100)	34 (100)
Total	160 (100)	147 (92)	151 (94)

¹Percent values are in brackets.

(11). The encouraging results obtained with this analytical scheme, and the need for greater method flexibility has led to an international I.C.M.S.F. evaluation of the refrigeration approach.

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TABLE 13. Recovery with prolonged incubation of selective enrichment media.

	Sample material	Preenrichment/enrichment	Percent recovery after			
			24 h	48 h	72 h	96 h
<i>Preenrichment</i>						
Edel (15)	Minced meat (artificially)	BPW/MK TBG ₄₃	90	95		
	Minced meat (naturally)	BPW/MK TBG ₄₃	83	92		
Edel (16)	Animal feed	BPW/MK TBG ₄₃	95	100		
Harvey (24)	Sewage water	BPW/MK TBG ₄₃	64	100		
Vassiliadis (57)	Pork sausage	BPW/MK TBG ₄₃	52	64		
<i>Direct enrichment</i>						
Carlson (5)	Animal feed	NA ¹ /SBGS ₃₇	58	53		58
		NA/SBGS ₄₃	84	87		82
Kampelmacher (32)	Minced meat	NA/MK TBG ₄₃	75	100		
Edel (14)	Minced meat (artificially)	NA/MK TBG ₄₃	99	100		
	Feces (artificially)	NA/MK TBG ₄₃	80	99		
Edel (15)	Minced meat (artificially)	NA/MK TBG ₄₃	27	52		
	Minced meat (naturally)	NA/MK TBG ₄₃	56	69		
Grunnett (22)	Sewage water	NA/MK TBG ₄₃	60	80	95	100
Kafel (31)	Meat filtrate	NA/MK TBG ₃₇	36	34		
		NA/MK TBG ₄₃	44	59		

¹NA = Not applicable.

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(Mackey Article Citing D'Aoust)

The effect of sublethal injury by heating, freezing, drying and gamma-radiation on the duration of the lag phase of *Salmonella typhimurium*

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The duration of the lag phase of *Salmonella typhimurium* surviving heat, freezing, drying and gamma-radiation was used to indicate the time needed to repair sublethal injury. Following equivalent lethal treatments, heat and freeze-injured cells needed longer to repair than those injured by drying or gamma-radiation. Measurement of repair on membrane filters showed that in a heat-injured population having a lag time of 9 h, some individual cells needed up to 14 h to recover maximum tolerance to 3% NaCl.

Examination of particular foods for salmonellas is an important part of efforts to control food-borne salmonellosis, and hence much attention has been given to improving the performance of isolation techniques and reducing the time taken for analysis (D'Aoust 1981; Hartman & Minnich 1981; Moats 1981). The isolation of these organisms from foods is a lengthy process entailing pre-enrichment in a non-selective broth to allow repair of sublethally injured cells followed by selective enrichment, usually in tetrathionate or selenite broths, plating on diagnostic indicator media and, finally, biochemical and serological confirmation of the isolates (Harvey & Price 1979).

Pre-enrichment or resuscitation treatments can improve dramatically the frequency of isolating salmonellas, especially in foods preserved by heating, freezing, drying and other processes (North 1961; Edel & Kampelmacher 1973). The usual pre-enrichment treatment consists of incubating the food sample for 16–24 h in a non-selective medium such as lactose broth or

buffered peptone water. Although this works well for many foods, it is time-consuming and can lead to false negative results if the sample contains large numbers of competing micro-organisms (Silliker *et al.* 1964).

Attempts to shorten pre-enrichment times, e.g. to 6 h, have met with limited success, presumably because some injured salmonellas need longer to recover resistance to selenite or tetrathionate and/or to multiply to levels which increase the likelihood of detection (D'Aoust & Maishment 1979). There is little systematic information on the effect on repair times of different physical stresses of varying intensity though this is clearly relevant when deciding on appropriate resuscitation treatments for examining foods preserved by different processes.

Previously Mackey & Derrick (1982) showed that recovery of resistance to selective agents was influenced by the composition and physical state of the medium but that by the end of the lag phase there was little difference in counts between media. In this paper the lag times of injured bacteria were used to assess differences in repair time following exposure to different physical stresses of varying intensity.

Materials and Methods

ORGANISM

Salmonella typhimurium LT2 (NCIB 10248) was subcultured every 2 months on digest agar slopes incubated at 37°C. The slopes were kept at 4°C between subcultures.

MEDIA

Digest agar was prepared from Hartley's Digest Broth (Southern Group Laboratories, Hither Green Hospital, London, SE13) by the addition of 1.2% (w/v) agar (Lab M No. 2). Maintenance medium (MM) contained (g/l): Bacto Peptone (Difco B118) 1.0; NaCl 8.5; pH 7.0.

Tryptone Soya Broth (TSB, Oxoid CM129) was supplemented with 0.3% (w/v) yeast extract and solidified as required with 1.3% (w/v) agar (TSA). Tryptone soya broth plus pyruvate (TSBP) contained 0.1% (w/v) sodium pyruvate added as a concentrated filter-sterilized solution to the autoclaved and cooled broth. Tryptone soya agar plus pyruvate (TSAP) also contained 0.1% (w/v) sodium pyruvate, added to the molten agar before pouring the plates.

Where indicated, sterile stock solutions of selective ingredients were added to molten TSA, to the following final concentrations (% w/v): sodium hydrogen selenite 0.4 (TSASEL); sodium chloride 3.0 (TSAS); sodium deoxycholate 0.075 (TSAD). Growth media and sodium chloride stock solution were sterilized by autoclaving at 121°C for 15 min. Sodium deoxycholate stock solution was sterilized by filtration; sodium hydrogen selenite stock solution was made immediately before use and was not sterilized.

VIABLE COUNTS

Serial decimal dilutions were made in MM and quadruplicate 0.02 ml volumes of appropriate dilutions, delivered from 20 SWG stainless steel cannulae (Astell Ltd, Catford, London), were spread on quarter sectors of agar plates incubated at 37°C. Colonies were counted when maximum numbers had developed (24 h for uninjured cells, up to 3 d for injured ones).

MEASUREMENT OF LAG TIMES

Injured bacteria were inoculated into 200 ml volumes of TSBP contained in 1 l flasks and

incubated at 37°C in a shaking water bath set at 100 rev/min. Samples (1 ml) were removed at intervals for viable counting, and a growth curve of log₁₀ bacteria/ml against time was constructed.

A growth curve was fitted to the data using the Gompertz function which relates log bacterial number (y) to time of incubation (x) in the following way:

$$y = A + Ce^{-e^{-B(x-M)}}$$

where A is the lower asymptotic coordinate of y ; B is a slope parameter; C is the distance between upper and lower asymptotes and M is the x coordinate of the inflexion point. Curves were fitted using the computer programme MLP (Ross 1975). The lag time was estimated as the intercept of the tangent to the fitted curve at the inflexion point, with the lower asymptote, i.e. lag time $t_l = M - 1/B$. Standard errors were estimated from the first order partial derivatives of T_l with respect to M and B . The method of estimating lag is based on that described by Garrett *et al.* (1978) who fitted logistic curves to growth data. The Gompertz was preferred because it fitted our data better than the logistic (or generalized logistic) function.

MEASUREMENT OF REPAIR TIMES

Method 1. Differential counting during incubation in TSPB

Injured bacteria were inoculated into 200 ml of TSPB and incubated as described for the determination of lag times. Samples were removed at intervals and counted on TSAP and TSAS. Where indicated counts were also done on TSA, TSAD and TSASEL. Recovery of resistance to each selective medium was detected as an increase in count on that medium whilst counts on the reference medium, TSAP, remained constant.

Method 2. Repair on membrane filters

Measurement of repair of cells spread on membrane filters allows detection of slow-repairing cells whose presence would otherwise be masked by multiplication of those repairing more rapidly.

Injured bacteria were decimally diluted in MM and 0.1 ml volumes from each dilution were inoculated on membrane filters (Oxoid

N85/45UP) on TSAP plates incubated at 37°C. Twelve membranes were inoculated from each dilution and, at zero time and intervals thereafter, one membrane from each dilution was transferred to TSAS. Colonies were counted after 1–3 d incubation at 37°C; counts on membranes transferred to TSAS gave the number of cells resistant to 3% (w/v) NaCl at each sample time whilst counts on additional membranes incubated continuously on TSAP gave the total viable count.

INJURY TREATMENTS

Growth of organism

A 50 ml conical flask with 10 ml TSB was inoculated with a loopful of surface growth on a TSA plate, and incubated for 16–20 h at 37°C in a shaking water bath set at 100 rev/min. Cells (1.5 ml) from the resultant stationary phase culture were harvested by membrane filtration (Oxoid N85/45UP), washed twice with 5 ml of 0.1 mol/l potassium phosphate buffer pH 6.0 and resuspended in 5 ml of buffer.

Denser starting suspensions were used when the reduction in viable count was to be greater than about 5 log cycles, ie. in some gamma-radiation experiments. These suspensions were prepared by centrifuging 10 ml of culture (5000 g for 10 min) at room temperature, washing twice and resuspending in 1–5 ml of phosphate buffer.

Heating

One millilitre of washed cell suspension was added to 50 ml of pre-heated potassium phosphate buffer (0.1 mol/l, pH 6.0) contained in a 250-ml conical flask immersed up to its neck in a water bath set at 52°C. In some experiments cells were washed then heated in 0.1 mol/l Tris HCl (pH 7.2) containing 0.011 mol/l $MgCl_2$.

Frozen storage

The washed cell suspension was diluted 50-fold in 0.05% (w/v) NaCl and 1.5 ml volumes were dispensed in glass ampoules placed in a water bath containing polyethylene glycol set at –10°C. Ice formation was induced by touching the surface of suspensions with a cooled glass rod, then ampoules were sealed with rubber

bungs. Following varying periods of storage, suspensions were thawed by placing the ampoules in water at 37°C until the last crystal of ice had disappeared.

Drying

Cells were dried in bovine albumin (Sigma, A-4503). When reconstituted in MM, this dissolved readily to give a particle-free solution minimizing counting errors due to non-homogeneous distribution of bacteria. Other substances such as gelatin or milk powder were less satisfactory in this respect.

Sterile albumin powder was prepared by filter-sterilizing an aqueous solution, re-drying it in Petri dishes at 43°C and grinding in a sterile mortar. Washed bacterial suspension (2 ml) was added to 0.5 g of sterile power and, when the albumin had dissolved, 0.1 ml volumes were dispensed in sterile 1 oz bottles lightly plugged with cotton wool. The bottles were placed at 20°C either in a desiccator over saturated $MgCl_2$ (r.h. = 33%) or in an open container. Dried cells were rehydrated by adding 9.9 ml of MM to the bottles.

Gamma-radiation

Washed cell suspensions contained in 1 oz screw-capped bottles were exposed to gamma-radiation from a ^{60}Co source at a dose rate of approximately 160 krad/h.

Results

RELATIONSHIP BETWEEN LAG TIME AND TIME TO RECOVER RESISTANCE TO SELECTIVE AGENTS

Ingredients commonly used in selective media inhibited heated cells to different degrees, but resistance to each was regained by the end of the lag phase (Fig. 1). TSAS was the most inhibitory medium tested and was used in further investigations of the relationship between lag time and time to recover resistance to selective media. Resistance to TSAS of heat- and freeze-injured cells was never fully restored until the end of the lag phase, irrespective of the length of that lag. Gamma-irradiated and dried cells were less sensitive to TSAS and sometimes recovered full resistance before the end of the lag phase (data not shown).

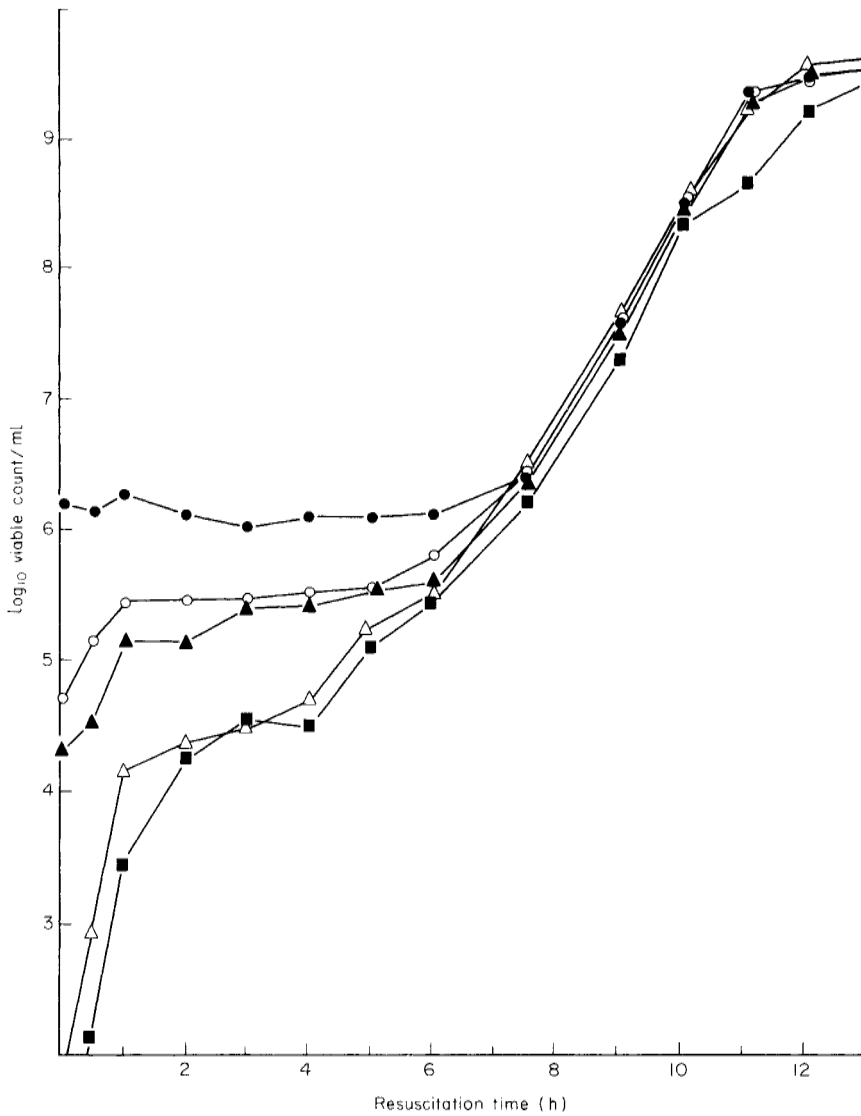


Fig. 1. Recovery of resistance to different selective agents during resuscitation of heat-injured *Salmonella typhimurium*. Cells were heated at 52°C for 30 min in 0.1 mol/l potassium phosphate pH 6.0, then diluted ten-fold in TSBP and incubated at 37°C. Samples were removed at intervals and viable counts determined on: ●, TSAP; ○, TSA; ▲, TSAD; △, TSEL; ■, TSAS. Survival after heating, measured on TSAP was 30%.

The presence of cells having repair times longer than the lag time of the population was investigated by monitoring repair of cells spread on membrane filters and simultaneously measuring the lag time of the same population in TSBP. Figure 2 shows that, following heating at 52°C in phosphate buffer to a survival level of 8.26%, the time needed to recover maximum tolerance to 3% NaCl (ca 14 h) was longer than the lag time (9 h). (The maximum count on

TSAS was lower than on TSPA because 3% NaCl is slightly inhibitory even to fully repaired cells.)

LAG TIMES OF *Salmonella* INJURED BY DIFFERENT STRESSES

Lag times of salmonellas injured by heating, freezing, drying and gamma-radiation were

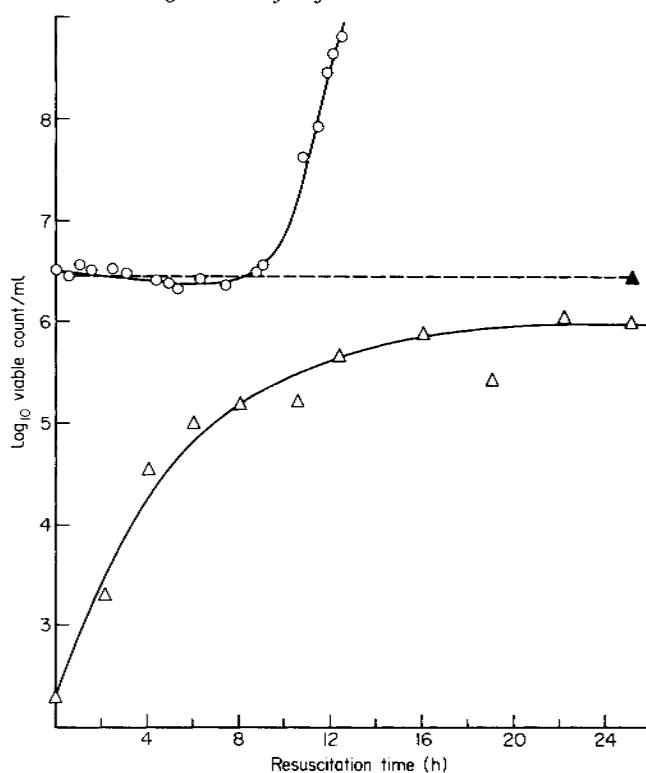


Fig. 2. Comparison of lag time with time needed to recover maximum resistance to 3% NaCl. Cells were heated at 52°C for 60 min in 0.1 mol/l potassium phosphate pH 6.0. ○, Lag time of the injured population in TSBP; Δ, restoration of salt tolerance monitored by the membrane filter method as described in Materials and Methods; —▲—, count of injured cells incubated continuously on membrane filters on TSAP.

measured as a means of detecting gross differences in repair times. To compare the effects of equivalent lethal treatments, lag times are plotted against percentage survival (Figs 3 and 4).

Heated and frozen cells both showed an initial phase of rapidly increasing lag with decreasing survival, followed by a phase when further reduction in viable numbers produced smaller increases in lag times of survivors. The curve for frozen cells showed a plateau region representing a maximum lag of 6–7 h.

Dried and irradiated cells showed much smaller increases in lag with decreasing survival. Lag times of irradiated cells increased continuously but slowly as viable numbers decreased, reaching a value of 3–4 h at 10^{-5} % survival. Dried cells stored at 20°C in an open container or over saturated MgCl_2 (r.h. = 33%) showed little change in lag time over the survival ranges examined.

The effect of heating in different media on lag time is shown in Fig. 5. Lag times increased

more rapidly during heating in phosphate buffer than in Tris-magnesium buffer though there was little difference in the lethal effect of the two treatments (survival after 15 min heating was 41 and 61% in phosphate and Tris-magnesium, respectively).

Discussion

Sublethally injured bacteria inoculated into growth media undergo a prolonged lag phase during which time injury is repaired and the ability to grow on selective media restored. We found that lag times of salmonellas injured by different types of stress were a convenient means of detecting differences in repair times. Before discussing the effects on lag of the different treatments, the relationship between lag time and time to recover resistance to selective agents should be considered, since this is the important practical criterion of repair.

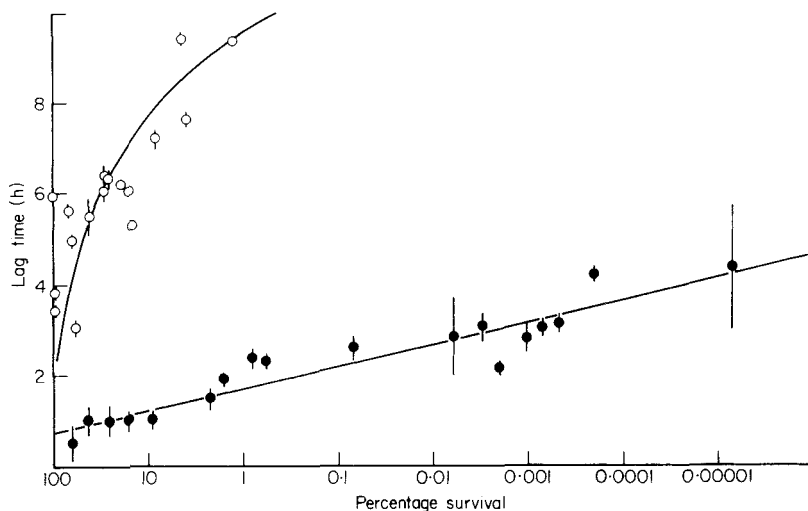


Fig. 3. The effect of heat or gamma-radiation on the duration of the lag phase of *Salmonella typhimurium*. Lag times were determined at different levels of survival: ○, following heating in 0.1 mol/l phosphate buffer pH 6.0 at 52°C; ●, following gamma-irradiation from a ^{60}Co source.

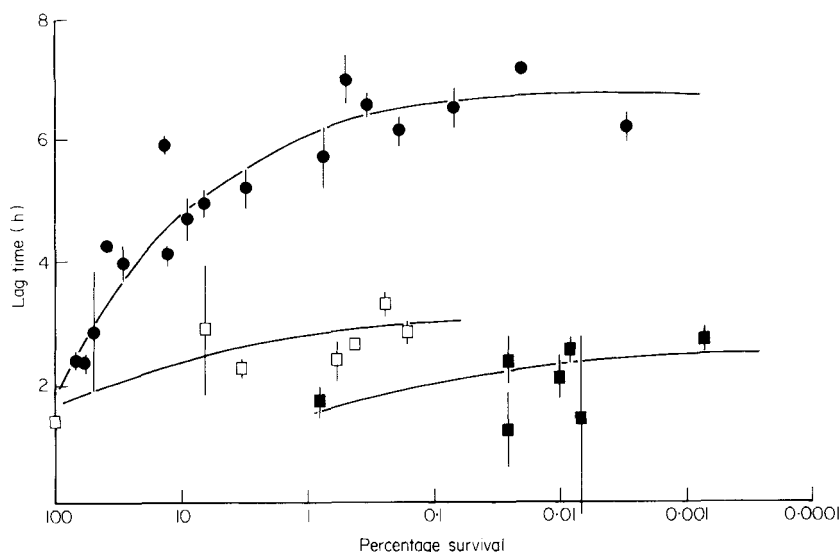


Fig. 4. The effect of freezing or drying on the duration of the lag phase of *Salmonella typhimurium*. Lag times were determined at different levels of survival: ●, following frozen storage at -10°C in 0.05% NaCl. Following storage of dried cells at 20°C in: □, an open container; ■, saturated MgCl_2 (r.h. = 33%).

Van Schothorst & van Leusden (1975) showed that *Salmonella utrecht*, injured by drying, recovered resistance to taurocholate before resistance to ox-bile and recommended that the influence of different selective media on repair time be investigated further. In this paper and previously (Mackey & Derrick 1982) differences were also found in recovery measured on

different selective media but by the end of the lag phase those differences had largely disappeared. Thus the estimated time for complete repair of injury will vary only slightly with different selective media.

Populations of injured cells are heterogeneous and contain individuals with varying degrees of injury (van Schothorst & van Leusden 1975).

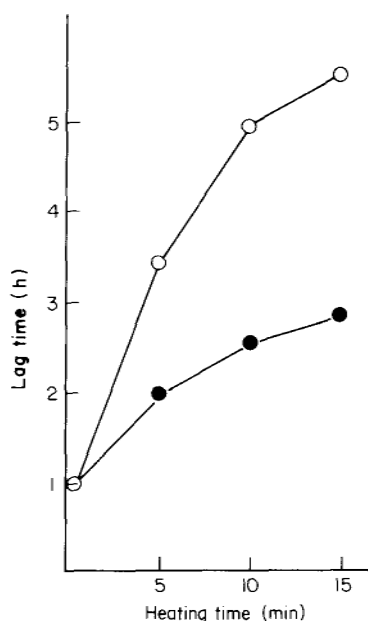


Fig. 5. Effect of the heating medium on the extension of the lag phase of *Salmonella typhimurium*. Lag times were determined following heating at 52°C in: ○, 0.1 mol/l potassium pH 6.0; ●, 0.1 mol/l Tris HCl plus 0.011 mol/l $MgCl_2$ pH 7.2.

The measured lag time of such populations may therefore reflect the lag times of the least severely injured individuals which are first to repair injury and to begin multiplication. The time to recover maximum salt tolerance in individual cells spread on membrane filters was longer than the lag time of the population as a whole, suggesting that lag times may underestimate the time needed for repair of all injured cells. However, this difference may have been due to different rates of repair on solid and in liquid media.

Wide differences in lag time were observed, depending on the type of stress and its severity. Heated, frozen and irradiated cells all showed increasing lag times as survival decreased but the rate of increase in lag was much more rapid in heated and frozen cells than in those irradiated. Dried cells showed only a very slight increase in lag time as viable numbers declined. Clearly, there is no direct relationship between lethal and sublethal injury for the different treatments.

Preliminary results showing longer lag times of cells heated in phosphate buffer, which causes extensive ribosome breakdown, compared with those heated in Tris-magnesium buffer, which

conserves ribosome structure (Tomlins & Ordal 1976), suggest that the extent of ribosome breakdown may be a major determinant of repair time.

Although comparative data are scarce, the effects of individual stresses on lag time have been studied in some detail. Several early workers described delayed development of colonies on agar following heat treatment and attributed this to extended lag (Nelson & Baker 1954; Lawton & Nelson 1955; Heather & Vanderzant 1957). The length of delay in colony formation was proportional to the severity of the heat stress (Anagnostopoulos *et al.* 1966).

Repair of sublethal heat injury in salmonellas has been studied extensively (Tomlins & Ordal 1976) but typical lag/repair times of 4–6 h are shorter than the maximum we observed. This presumably reflects differences in the degree of injury in surviving populations, as those experiments on repair of sublethal injury were performed under conditions of minimum stress designed to produce sublethal but little lethal injury.

Much longer lag times have been observed in Gram negative bacteria following heat treatments which significantly reduced viable numbers (Hershey 1939; McCoy & Ordal 1979). An extreme case of prolonged lag was described by Dabbah *et al.* (1969) when a *Pseudomonas* sp. heated until only a few survivors remained, then incubated in broth at 20°C, needed 48–72 h to recover ability to grow on solid medium. Gram positive bacteria also exhibit extended lags following heat treatment, the length of lag being proportional to the severity of heating (Kaufman *et al.* 1959; Jackson & Woodbine 1963; Payne 1978).

It is harder to generalize about the effects of freezing on lag times. Hartsell (1951) observed no lag with defrosted *Escherichia coli* following storage in liquid egg at -9°C and Squires & Hartsell (1955) obtained a complex pattern of decreasing, then increasing lag with *Esch. coli* defrosted after increasing periods of storage at -9°C in yeast extract veal infusion. Most workers, however, have found only increased lag times with frozen and thawed cells (Postgate & Hunter 1963; Ray & Speck 1973). We found that lag times of *Salmonella* increased with increasing duration of frozen storage, reaching 6–7 h which was not exceeded as viable number declined further. This may reflect a steady state between lethal and sublethal injury.

An increase in lag of up to 2 h occurred with *Esch. coli* B following gamma-irradiation to 10% survival (Alper 1957). *Esch. coli* B is not entirely typical in its response to radiation because it contains the *lon*⁻ allele resulting in post-irradiation filament formation. Nevertheless the rather small increase in lag was similar to that of irradiated salmonellas.

Salmonella utrecht in freshly prepared dried milk powder recovered resistance to tetrathionate within 6 h, but repair time increased to 6–18 h on storage of the powder (van Schothorst & van Leusden 1972, 1975). Lag times of *Salm. typhimurium* dried in albumin were short even at low levels of survival, implying short repair times and mild injury. We cannot explain the difference between these results. Drying is a complex stress, bacterial survival being influenced by numerous variables operating during drying, storage and rehydration (Strange & Cox 1976; van Schothorst *et al.* 1979). These same variables may also affect the relative proportions of severely and slightly injured cells amongst survivors, and hence lag times.

This work showed that the severity of sublethal injury, indicated by the time needed to repair that injury, is different in heated, frozen, dried and gamma-irradiated cells and is not proportional to the percentage survival measured on complex medium. This implies that during exposure to stress, lethal and sublethal events can occur independently and at different rates depending on the nature of the stress. A comparison of the effects of different treatments on specific cell components might clarify the relationship between lethal and sublethal injury.

Measurement of lag times, though not providing precise information about the time needed to recover resistance to particular selective agents, does allow useful and convenient comparisons of repair time following exposure to different stresses. Lag times of cells exposed to specific food processing treatments would provide a guide to whether long or short resuscitation treatments were necessary when examining particular foods. Under laboratory conditions some injured cells needed up to 14 h to repair injury. Cells in food having similar long repair times would explain why prolonged pre-enrichment is sometimes necessary to ensure maximum recovery of salmonellas.

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