J. Hyg., Camb. (1962), **60**, 193
Printed in Great Britain

A discussion of the dynamics of salmonella enrichment

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(*Received* 16 *October* 1961)

INTRODUCTION

As in other biological phenomena, the course of a fluid enrichment culture is determined, as this paper will amply endorse, by the interaction of a complex of variables. To observe the influence of one, the others have to be held constant.

In a previous publication (Jameson, 1961), to which frequent references will be made, a concept of enrichment dynamics was introduced by aid of theoretical simplification of variables. The purpose of this paper is to widen this concept and to discuss other factors which bear on enrichment dynamics.

It is well known that, after inoculation to broth, an organism may pass serially through various phases, a lag phase, a phase of logarithmic multiplication, a so-called stationary phase, and a phase of decline. When logarithmic multiplication has led the density of bacterial population to rise near to a level, defined by Bail (1929) as molar concentration, logarithmic multiplication ceases. The attainment of molar concentration is therefore, as might be expected, an important turning point in enrichment dynamics, which might well end at this juncture, were it not that the so-called stationary phase belies its name. Evidence will be presented that after molar concentration is reached, a pattern of dynamics continues to unfold, though in obedience to somewhat different laws. Separate consideration will therefore be given to a first phase of enrichment which takes place during the build up to molar concentration and a second phase which follows after. Later, use of high temperature incubation, of secondary enrichment, and of solid media, will be discussed.

DISCUSSION

First-phase enrichment

When two intestinal organisms, which do not mutually interact by colicines or bacteriophage, are inoculated together into a liquid medium, each organism normally follows at first a growth pattern similar to that which would have followed from a similar inoculum in the same medium in the absence of a competitor. Neither organism normally exhibits its awareness, to any appreciable degree, of the other's presence, until the bacterial density of one or other organism has risen to a level near to the molar concentration, when *both* organisms end their rapid multiplication (Fig. 1 and Appendix p. 207).

If a selective agent is added to the liquid medium, the growth-pattern of either or both organisms may be influenced in one or more of the following ways:

Bactericidal activity

Either organism may exhibit a progressive decline in its viable count.

Bacteriostatic activity

This may be absolute, that is the lag period of one or both organisms may be indefinitely prolonged, or relative, that is to say that a lag period may be lengthened by the selective agent.

Reduction in growth rate

The generation time of either organism may be lengthened.

Diminution of molar concentration

Visual confirmation of this effect may be seen in the lesser turbidity of a 24 or 48 hr. liquid culture compared with the turbidity of a like culture in the absence of the selective agent. Should there be greater than a hundred fold diminution in molar concentration, bacterial turbidity will usually not be visible. Complete absence of turbidity may therefore be a misleading indication of bacteriostasis.

Diminution of viability

The minimum inoculum of either organism for survival, or for the initiation of growth in the medium, may be increased by the selective agent. This effect may differ from bactericidal activity in degree only; that is, an organism which is mainly susceptible to the bactericidal activity of a selective agent may contain a proportion of resistant variants which are selected and multiply out in the selective medium.

Possible modes of action of a selective agent have for convenience been listed above as differing degrees of growth inhibition, but converse effects may also produce selective action. What concerns us is the algebraic difference between the effect of a selective agent on the organism we hope to isolate, let us say a salmonella, and on its main competitor. A selective agent which influenced the ratio of generation times of a salmonella and competitor by *lengthening* the generation time of the competitor might well produce selective activity almost indistinguishable from that of another which influenced the ratio in the same direction by *shortening* the generation time of the salmonella. As long as the sign of the algebraic difference between the two effects is favourable to a salmonella, a minor degree of inhibition of a salmonella by a selective agent is usually of no consequence. For example, moderate diminution of molar concentration is very easily compensated for simply by a small increase in the size of the inoculum to solid media and to secondary enrichment, and, when dilutions of enrichment cultures are plated out, as advocated by Moore (1950), by plating out lower dilutions. Similarly a not unduly large increase in the lag period or generation time induced in a salmonella by a selective agent might pass unnoticed in an overnight culture, since under more favourable conditions, a minimal inoculum of salmonellae nears molar concentration within 6 or 7 hr. Diminution of viability of a salmonella by a selective agent, cannot, on the other hand, be tolerated lightly, as a greater number of salmonellae must then be present in a sample before isolation becomes possible. Certain balanced tetra-

thionate media are commendable in this respect as some strains of salmonellae have been shown to be equally viable in them as in nutrient broth (Jameson, 1961). Though in the cultural isolation of *Mycobacterium tuberculosis* from sputum, a short exposure is given to an agent (the homogenizing agent) bactericidal to *M. tuberculosis*, the isolation of salmonellae is so different a proposition that it is difficult to imagine circumstances in which use of a selective bactericidal agent might be a method of election of salmonella isolation. Salmonellae are however relatively resistant to some bactericidal agents, for example concentrated Teepol at the pH range of 8–9 (unpublished).

In any fluid enrichment medium the lag period of a salmonella may be longer or shorter than that of its main competitor. This also holds good for the generation time. The two organisms can thus co-exist in a medium in four possible combinations of these characteristics. When both the lag period and the generation time of a salmonella are greater than that of its competitor, no enrichment of the salmonella can occur during the first phase; though during this phase the absolute number of salmonellae may increase, there must necessarily be a decline in their relative numbers. In such circumstances the successful outcome of an enrichment will depend on the efficacy of the solid selective medium used for plating and on events which take place during the second phase of enrichment. Conversely, when the salmonella has a growth advantage in both of the above respects, first-phase enrichment must necessarily occur. This does not mean that plating at the end of the first phase of enrichment will necessarily reveal the presence of salmonellae. In apparent paradox, in this example, the likelihood of detection of salmonellae by plating increases directly with the number of generations of multiplication made possible to the main competitor (Jameson, 1961). There remain two combinations of characteristics in which the salmonella is at advantage in one of two of the respects. Consideration of the factors which influence the course of first-phase enrichment in these two examples may serve not only as an exercise for the intellect but also to underline the influence of technical details other than the appropriate choice of media.

Combination 1. *Salmonella has longer lag period but shorter generation time*

Maximal first-phase enrichment would be obtained from the smallest possible inoculum (containing one viable salmonella) into the largest practicable bulk of enrichment medium. In practice this may be achieved by diluting the inoculum, see Lendon & Mackenzie (1951).

Combination 2. *Salmonella has shorter lag period but longer generation time*

First-phase enrichment would be difficult. The shorter lag period could be exploited by repeated subculture, but adequate time at molar concentration would have to interpose between each passage, to enable the competitor to re-establish itself in a resting phase and thereby regain its lengthened lag period. At every passage relatively large inocula would have to be transferred (perhaps equal to $\frac{1}{8}$ or $\frac{1}{4}$ of the total volume of the enrichment) in order to minimise the numbers of

generations of multiplication which followed each exploitation of the lag period advantage.

It is well known that alterations in pH and eH influence the selective activity of dyes and other selective agents, but the degree to which differences in eH (attributable to such factors as depth of enrichment fluid, presence of a tightly screwed cap, etc.) exert this influence may not be as well recognized. For example, oxygen continuously bubbled through nutrient broth may elevate a molar-concentration 10- to 30-fold.

In the writer's experience of selective agents used in the isolation of salmonellae, an increase in oxygen tension often reduces the concentration of a selective agent necessary to achieve a given selective effect. An illustration is a tetrathionate broth, dispensed in tubes to a depth of approximately $\frac{5}{8}$ in. which is more inhibitory than double quantities of the same medium dispensed in similar tubes. Similarly, no intestinal organisms would grow on the surface of a solid medium containing a concentration of selenite normally used in selenite broth. There are occasions on which differences in eH exert a converse influence, as in selenite broth (Moore, 1950).

Second-phase enrichment

Topley & Fielden (1922) showed that on continued incubation of broth cultures of faeces, a succession of dominant species occurs. The value of ordinary nutrient broth for salmonella enrichment from faeces was emphasized by Thomson (1955). Dixon (1959) showed that pairs of faecal strains of *Escherichia coli*, inoculated into broth in unequal proportions, tend to reach numerical parity on continued incubation. The tendency to equation increased with increasing length of incubation. McCoy (1962) counted the salmonellae in a series of polluted waters, and observed the comparative results from plating enrichments after 24, 48, 72 and 96 hr. incubation respectively. One of his two main findings was consistent with common bacteriological experience, namely that the more heavily a sample was infected with salmonellae, the earlier the enrichment culture became positive by plating. His other finding is less easy to explain. Perhaps due partly to the evolution of second-phase enrichment and partly to effects similar to those described by Smith (1959), if plating had been restricted to 96 hr. the majority of his most strongly positive specimens would have been missed. He thus showed that the optimal time of incubation of his enrichments was a function of the number of salmonellae present in the sample being examined.

To these varied observations the author will add one of his own. Relatively small inocula of various organisms were added to broth cultures of others already at molar concentration, that is, to fully turbid overnight broth cultures. Serial counts of the minority organisms were then made during continued incubation at 37° C. Pairs of organisms studied were *Salmonella litchfield* and *Esch. coli*, *Esch. coli* and *Salm. litchfield*, *Salm. typhi* and *Esch. coli*, and *Shigella sonnei* and *Esch. coli*. The growth curves are plotted in Fig. 2. In view of Dixon's findings, second-phase enrichment of the minority organisms in each of the four pairs is not very surprising, although the pairs were dissimilar, and in at least two of them the minority organism would have lost ground during *first-phase* enrichment in nutrient

broth. It is more noteworthy that in every case the growth curve of the minority organism was of the straight-line type which denotes logarithmic multiplication, until a count was reached of about 10 million/ml. These curves differ from normal growth curves of the same organisms in the gradients, that is, under the conditions of these tests, the generation times were lengthened, especially in the case of *Shigella sonnei*. (It should be added that several further attempts to demonstrate multiplication of *Shigella sonnei* under similar conditions gave variable results. It was later found that this strain of *Esch. coli* is capable of producing a colicine to which this strain of *Sh. sonnei* is sensitive.) Other points of difference are in the lower counts at which the curves flatten out, and in the extended lag period which followed a *small* inoculum of *Esch. coli*.

In Fig. 3, growth curves have been plotted of *Salm. litchfield* and *Esch. coli*, simultaneously inoculated in highly unequal proportions, to broth at 37° C. This figure has been included because of the clear way in which an intermediate stage has been shown, lasting approximately 4 hr., between first- and second-phase enrichment of the minority organism (*Esch. coli*). Owing to the very heavy inoculum of salmonellae, first-phase enrichment of *Esch. coli* was greatly shortened.

One cannot review these varied observations on second-phase enrichment without trying to explain them. We know that, when in stationary phase following attainment of molar concentration, a clone maintains a steady population by replacing deaths of cells by restricted multiplication of others. We also know that when in this stationary phase, the lag period (demonstrable on subculture) is lengthened. Thus, when clones of two species co-exist, a dominant clone which has reached molar concentration with a minority organism which has not, the latter organism will, other things being equal, be at an advantage over the former in respect of its shorter lag period: also, by virtue of its small numbers, the minority organism will make a smaller contribution to the total cell deaths, each of which it is better equipped to replace than its competitor with a lengthened lag period. This can explain why equalization in numbers occurs. Implicit in this hypothesis is the assumption that environmental changes which induce a dominant organism to enter the resting phase do not produce equal effects on minority species also present in the same environment. One has therefore to assume an element of specificity, in some way related to the population-density of the homologous organism. If lengthening of the lag period also increases progressively with duration in the resting phase, the lag period of the minority organism might never quite catch up with that of its predecessor, so that the process causing equalization might continue beyond the point of numerical parity. Perhaps some other aspects of ageing, in its wider sense, also contribute when a formerly dominant clone is completely superseded by another.

Influence of high temperature incubation

Harvey & Thomson (1953) increased their isolation rate of salmonellae from selenite enrichments of faeces by raising the temperature of incubation. In a particular fluid medium of defined pH, nutrient capacity, and selenite concentration,

they found 43° C. an optimal temperature for 24 hr. salmonella enrichment. Dixon (1959) with a different medium, advocated enrichment of faecal strains of *Esch. coli.* at 44° C. When incubation temperature is raised above a certain point, a nutrient medium which at a lower temperature supports active bacterial multiplication quite suddenly becomes bactericidal. In nutrient broth of determined pH and eH, the temperature at which this transition occurs is considerably influenced by the nutrient capacity of the medium, that is, by its content of amino-nutrients and fermentable sugar (see Table 1). As should be expected, an increase in the concentration of any inimical factor such as an inhibitory selective agent, or a removal of pH further from the optimal for the organism in question, lowers this temperature of transition. As the optimal incubation temperature for salmonella isolation must lie below the temperature of transition, factors which influence the latter must generally exert a similar influence on the former. In a given medium incubated at a given temperature, there is an optimal concentration for any given

Table 1. *Multiplication and death of Salmonella typhi-murium incubated in fluid media at* 46° C.

Inocula of approximately 300 organisms were added to media of differing nutrient capacity and incubated overnight at 46° C. Counts were then made, and the temperature of incubation was reduced to 37° C. The variables in the media were the content of amino-nutrients, and glucose.

Glucose (%)	Amino-nutrient (%) 6	2	0·6
3	M	M	=
1	M	=	=
0·3	M	S	S

M, Active multiplication; S, total sterilization; =, intermediate effects, survival or limited multiplication.

The amino-nutrient consisted of equal parts Lab. lemco and Evans's peptone. Thus media containing 6 % amino-nutrient contained 3 % each of Lab. Lemco and Evans's peptone. The fixed constituents of the medium were 0·5 % NaCl, M/60 KH_2PO_4, a small quantity of added chalk, and a pH of 7·2. All tubes were similar and contained the same volume of medium.

selective agent for the isolation of a particular organism. When the concentration of the selective agent is fixed, as in Harvey & Thomson's observations, there must be an optimal temperature of incubation. The fact that 43° C. was optimal for the selenite medium used by Harvey & Thomson suggests that when this medium is used at 37° C. for salmonella isolation, its content of selenite may be less than optimal. A fixed concentration of a selective agent which exerts a bacteriostatic influence on a competitor at 37° C. may at a higher temperature, compatible with salmonella multiplication, exert a *bactericidal* effect. Moderate elevation above 37° C. of incubation temperature does not necessarily reduce the growth rate of a salmonella and may *increase* it. Identical inocula of *Salm. litchfield* were added to 20 ml. quantities of nutrient broth in 1 oz. screw capped bottles pre-heated to 37°, 41°, and 44° C., respectively. A count was made immediately after on one of these broths by a modification of the Miles & Misra (1938) technique. The three

bottles were held at their respective temperatures for 24 hr, and further counts were made on the three cultures at intervals of 1, 2, 4, 6, 8, and 24 hr. (Table 2). When growth curves were plotted it could be seen that the lag period of the salmonella was rather greater than 45 min. both at 37° C., and at 44° C., but at 41° C. this lag was reduced by about 15 min. The growth rate was most rapid at 41° C., and slowest at 44° C. Ignoring any differences which may have been present between the viable and total counts, calculation of the generation times during logarithmic multiplication of the salmonella at the three temperatures, gave figures of 17·3, 17·9 and 19·8 min. at 41°, 37°, and 44° C. respectively. These relatively small differences were sufficient to produce an eightfold difference in the viable counts

Table 2. *Showing serial log counts on Salmonella litchfield incubated in broth at temperatures of* 37°, 41°, and 44° C. *respectively*

Incubation time (hr.)	Temperature of incubation		
	37° C.	41° C.	44° C.
0	1·89	(1·89)	(1·89)
1	2·04	2·35	2·11
2	3·12	3·33	2·97
4	5·12	5·44	4·83
6	7·08	7·58	6·68
8	8·40	8·11	7·95
24	8·59	8·26	8·20

Anti-logarithms of the figures shown above express counts/ml.

after 6 hr. incubation. It will also be noted that the molar concentration of the salmonella in broth was highest at 37° C., and lowest at 44° C. The size of an optimal inoculum for plating from an enrichment culture to a given solid medium must be influenced by the level of molar concentration in the enrichment. Thus, when similar inocula are plated from enrichments incubated at two different temperatures, they cannot both lie within the optimal inoculum range if there is a gross difference between the respective molar concentrations. This factor, if overlooked, might introduce an unintended bias when experiments are conducted on the lines of that by Harvey & Thomson (1953), and also when a comparison is undertaken between two quite different selective enrichment media incubated at the same temperature.

In view of what has been said, bacterial populations incubated at high temperatures must have a lesser tendency to become static than at 37° C., at which temperature there is a wider threshold between the conditions which promote bacterial multiplication and those which lead to bacterial death. Bactericidal influences will apply over a wider field at higher temperatures and exert their effects more rapidly. (For example, a one-quarter strength Ringer solution of neutral pH, comparatively inert at 37° C. to intestinal organisms, was at 44° C. found quite strongly bactericidal to a wide range of intestinal organisms, including salmonellae. Second-phase enrichment also may proceed at a greater speed at higher temperatures. Investigation of enrichment at temperatures above 37° C. in sufficiently

stable selective media, as pioneered by Harvey & Thomson, might well lead to improved techniques, though in such an investigation many variables would have to be taken into account. A selenite medium containing lauryl sulphate (Jameson, 1961) might perhaps be worthy of such trial.

Secondary enrichment

An advantage which may be gained by transferring a relatively large inoculum from an incubated primary enrichment to a further fluid medium has been discussed previously (Jameson, 1961). Transfer to a *different* fluid medium, when the two media are not incompatible,* has theoretical advantages in that a competitor which thrives in a primary enrichment may be later suppressed in a secondary one. Primary enrichment in medium A (Jameson, 1961) followed by secondary enrichment in medium B, has in the author's laboratory given results which seem to compare very favourably in all respects with more conventional methods.

Except when subculture is carried out soon after molar concentration has been reached in the primary culture, secondary enrichment must result in an alternation between first- and second-phase enrichments. While a first-phase enrichment may be completed in a matter of hours, second-phase enrichment is a slower process which, when many species compete, may continue over several days. As was shown earlier, with certain combinations of organisms and media no first-phase enrichment is possible, though second-phase enrichment may take place. With other combinations, either first- or second-phase enrichment, *if carried far enough*, may lead to salmonella isolation. To exploit second-phase enrichment fully, incubation at 37° C. may have to be prolonged for at least 4 days, (McCoy, 1962). A single stage of first-phase enrichment is normally completed within about 8 hr. It is therefore possible to complete three serial first-phase enrichments in 24 hr. Even when serial enrichment is transferred at 24 hr. intervals, a plating from a secondary enrichment might well reveal after 48 hr. the presence of a salmonella which by second-phase enrichment might take 96 hr. to elicit from a primary culture.

Silliker & Taylor (1958) have drawn attention to the fact that soluble foods impair the selective action of selective media. It therefore seems logical, when culturing soluble foodstuffs for salmonellae, to exploit second-phase enrichment (which takes place in non-selective as well as in selective fluid media) to the full, by prolonging incubation as long as practicable. It would seem equally logical, if this were possible, to obtain effectual first-phase enrichment by diluting out the soluble foodstuffs but not the salmonellae. Serial secondary enrichment seems to offer a means. So long as the selectivity of the medium is not impaired by the foodstuff to a degree in which the salmonellae are *culturally at a disadvantage* (as salmonellae hold their own well in nutrient broth, this does not seem very likely), and so long as molar concentrations in the secondary enrichments are as high as that in the primary enrichment, there can be no dilution of the

* Mixtures of single strength tetrathionate and selenite broths containing between 0·5 and 95% of selenite broth are inhibitory. Mixtures well within the fringes of this range are bactericidal to salmonellae.

salmonellae no matter how many consecutive serial enrichments are made. Suppose for example that 1 ml. of enrichment culture were transferred twice successively through 20 ml. quantities of secondary enrichment medium, then, when the conditions stated above were fulfilled, 1 ml. of the incubated final enrichment could contain no fewer salmonellae (probably many more) than were contained in the initial 1 ml. inoculum from the primary enrichment culture. This procedure would dilute the concentration of soluble food over 400 times, more than sufficient to nullify any undesirable effect previously exerted by it.

Different samples containing salmonellae are subject to great variation in growth potentialities of the competing species present, and in their relative numbers proportionate to the salmonellae. It is therefore not in the least surprising that a particular schedule of enrichment, which may give good results with some samples, may fail with others (Table 3). The table does not reveal any advantage

Table 3. *An analysis of positive results from platings for salmonellae, following various schedules of enrichment, from fifty-one samples of liquid whole egg received for examination while this paper was in preparation*

Sample no.	Total period of incubation							
	24 hr.	48 hr.		72 hr.				96 hr.
	P1	P2	P1S1	P3	P2S1	P1S2	P1S1T1	P4
1	−	−	−	−	−	−	++	−
2	−	−	+++	−	+++	+++	+++	−
10	−	−	−	−	++	++	−	−
14	−	−	+	−	++	++	−	−
17	−	−	−	−	−	+	−	−
30	+	−	+++	+	+++	+++	−	−
43	++	+	+++	−	+++	+++	+++	−
44	−	−	+++	−	++	+++	+++	−

P, Primary enrichment; S, secondary enrichment; T, tertiary enrichment; 1, 24 hr.; 2, 48 hr.; 3, 72 hr.; 4, 96 hr.

Approximately 70 ml. quantities of liquid egg were added to 350 ml. amounts of nutrient broth, incubated at 37° C., and plated after 24, 48, 72 and 96 hr. (primary enrichment). After 24 hr., 0·5 ml. of the primary enrichment was transferred to 25 ml. of medium B (Jameson, 1961) to make a secondary enrichment. After a further 24 hr., 0·5 ml. of the secondary enrichment was again transferred to 25 ml. of medium B (tertiary enrichment), and 0·5 ml. of the 48 hr. primary enrichment was transferred to a further bottle of medium B. At the times when the primary enrichment had completed 48 hr. and 72 hr. incubation respectively, the secondary and tertiary enrichments were also plated out. The platings which were made on bismuth sulphite agar, were graded for their richness in salmonellae as follows: +++, pure, or almost pure culture of salmonellae; ++, several or many salmonella colonies present; +, one or a few salmonella colonies present; −, salmonella not found.

gained from prolonging primary enrichment in broth of whole egg to 96 hr. though it does provide striking evidence of the value of secondary enrichment (in medium B). During the course of this trial, cognizance was taken of a high proportion of negative platings from the secondary enrichments which yielded *no bacterial growth*, and it was realized that while a large loopful of a 1/20 dilution of the primary enrichments in broth was near to an optimal inoculum for plating, two or

three undiluted loopfuls were more nearly optimal for the secondary enrichments in medium B. Owing to the relative purity of the growths from the platings from the secondary and tertiary enrichments, almost all the colony picks from these proved successful. Much time was however spent in making and pursuing a large number of colony picks, which were not salmonellae, from the primary platings. Omission of platings from the primary enrichments in this series would have saved much labour without reducing the number of isolations. An optimal inoculum from the tertiary enrichments was not ascertained, but must be as large, and probably larger than that from the secondary enrichments: for example, in the case of sample 30, an inoculum of 0·5 ml. was also plated from the tertiary enrichment, and though no bacterial growth was obtained from the plating of a single loopful, the larger inoculum yielded salmonella in pure culture. (This enrichment is shown as negative in the table, as use of such large inocula was not part of the routine method.) The reason why the tertiary enrichments failed in samples 14 and 30, the inocula to both of which must have contained salmonellae, is likely to be due to the dilution out of carry-over of appropriate organic material derived from the whole egg (Jameson, 1961). It has since been shown that the viability of some strains of salmonellae may be diminished in medium B *in the absence* of suitable added organic material. Media which contain bismuth sulphite are liable to become either more, or less inhibitory, on storage. Medium B becomes more inhibitory, and the author's practice has been to discard this medium after 24 hr. storage at 4° C. In this limited series, use of tertiary enrichment in medium B resulted in one additional salmonella isolation; whether or not tertiary enrichment is justified as a routine procedure in the examination for salmonellae of whole egg remains to be proved. Secondary enrichment can undoubtedly be highly rewarding.

Use of solid media

It is convenient to introduce the following discussion by doing counts by Miles & Misra's method, on overnight broth cultures on *Esch. coli* and of a salmonella, on a selective and a non-selective solid medium. The media chosen were deoxycholate citrate agar and Teepol agar, which latter medium is equally non-inhibitory to both species. Typical findings from such counts are set out in Table 4.

It will be seen in the table that on the non-selective medium (Teepol agar), confluent growth of *Esch. coli*, covering circular discs of 17 mm. diameter, resulted from inocula of 45,000 organisms, an inoculum-density of approximately 200 *Esch. coli* per square mm. of medium surface. Differently expressed, confluent growth occurred when the average area of medium occupied by each individual *Esch. coli* was equal in area with a square with sides of approximately 70 μ. (This may be compared with the space occupied by an organism at molar concentration in broth. When the count is 500 million/ml. each organism occupies an average volume equal to a cube with sides of approximately 13 μ).

As distinct from fluid media, on the surface of solid media *Esch. coli* is dominant in growth over salmonellae and shigellae. That is to say, if on any 70 $\mu \times 70$ μ area of Teepol agar medium surface one growth-viable *Esch. coli* is in competition for growth with any number of salmonellae or shigellae, growth of the former species

normally occurs, mainly to the exclusion of the latter. Qualification of this statement is necessary at the edges of a zone of confluent growth; here one or more salmonellae or shigellae which have been deposited within areas of domination by *Esch. coli* may also have access, uninterrupted by the latter species, to contiguous areas of medium. Under such conditions a half colony or rim of salmonella or shigella growth develops, often to be exploited when subcultures are made from solid media. It can also be seen in Table 4, that when viewed by reflected light, the smoothness of surface growth of *Esch. coli* on Teepol agar increased progressively with inoculum-size, up till the 10^{-1} dilution. It is as if the surfaces were composed of tiles of increasing compactness until a minimum size of tile was reached. It does not require a large flight of imagination to conceive that this may in fact have been the case, each tile having consisted of a clone of *Esch. coli*. This flight

Table 4. *Showing Miles & Misra counts on broth cultures of Escheria coli and Salmonella litchfield, plated on two different solid media*

Dilution of inoculum	*Esch. coli*		*Salm. litchfield*	
	Teepol agar	D-c agar	Teepol agar	D-c agar
Neat	PC (4,500,000)	PC (4,500,000)	PC (9,200,000)	PC (9,600,000)
10^{-1}	PC (450,000)	SD (450,000)	PC (920,000)	PC (960,000)
10^{-2}	C (45,000)	D 4·4	PC (920000)	C (96,000)
10^{-3}	SC (4,500)	D 0·6	C (9,200)	C (9,600)
10^{-4}	SD (450)	Nil	SC (920)	SC (960)
10^{-5}	D 45	Nil	SD (92)	SD (96)
10^{-6}	D 4·0	Nil	D 9·2	D 9·6
Average diameter of discs of confluent growth (mm.)	17	14	18	14

Letters in the columns denote the types of growth present (see key). The figures give the mean numbers of colonies which developed from single 0·02 ml. inocula. Where the colonial separation was insufficient for counting to be possible, the estimated number of organisms in the inoculum has been given in parenthesis.

D-c agar, deoxycholate citrate agar; P, polished appearance of growth by reflected light; C, confluent growth; SC, semi-confluent growth; SD, semi-discrete colonies; D, discrete colonies.

accepted, the discs of confluent growth arising from the neat and the 10^{-1} inocula each contained the same number of clones (450,000 by this titration), and each clone occupied an average surface area of approximately $(22\mu)^2$. It therefore seems possible that when the inoculum-density of *Esch. coli* on Teepol agar is greater than one per unit area ($22\mu \times 22\mu$)*, growth from the excess *Esch. coli* also is suppressed. This limiting inoculum-density is in a sense analogous to molar concentration.

The dominance of *Esch. coli* on a solid medium was made use of by the author when counting *Esch. coli* in the presence of a salmonella at molar concentration

* This calculation does not make allowance for irregularities in the distribution of the deposited organisms. It is also subject to considerable inaccuracy on account of the tenfold steps between the observations in the Miles & Misra count.

(Figs. 1, 2 and 3). The counts by a Miles & Misra method were made on Teepol agar. No difficulty was experienced in counting *both* organisms in a mixed culture containing over 1000 million salmonellae/ml. and approximately 50 *Esch. coli*/ml. (this count is not recorded elsewhere in this paper). Had a reciprocal ratio between the two organisms been present, a count of the salmonella would not have been possible except by the aid of a very efficient selective medium. Experimental confirmation of this dominance of *Esch. coli* may also be obtained by dropping 0·02 ml. of a mixed broth culture containing 500 or 1000 million salmonellae/ml. and 5 million *Esch. coli*/ml. on the surface of Teepol agar. This inoculum results in a disc of confluent growth mainly of *Esch. coli* except at the rim. In a non-selective medium permitting less luxuriant surface growth, the inoculum-density of *Esch. coli* to produce a similar effect might have to be somewhat increased.

On the basis of the inoculum-density necessary for confluent growth of *Esch. coli* on Teepol agar, it was calculated that each growth-viable *Esch. coli* exerts a dominance over a roughly $70\mu \times 70\mu$ area of medium. It is however surprising to what extent colonial development of salmonellae and shigellae may be restricted, on non-selective media, even in zones where discrete, though closely spaced, colonies of *Esch. coli* are present. It is therefore reasonable to add to this inner zone of dominance a surrounding zone of gradually diminishing dominance which extends over an area of 3 or 4 sq. mm. of medium surface.

In the case of selective solid media, the size of these zones of greater and lesser dominance by *Esch. coli* are lessened by the lesser exuberance of growth of *Esch. coli* on selective media. A more telling limitation may be imposed by selective media which reduce the growth-viability of *Esch. coli*. For example, it can be calculated from data supplied in Table 4 that while an inoculum-density of 200 *Esch. coli*/sq. mm. produced confluent growth on Teepol agar, the corresponding inoculum-density necessary on deoxycholate citrate agar was approximately 30,000/sq. mm.; that is to say, only one organism, in approximately 150, was growth-viable on the medium, at this inoculum-density of *Esch. coli*. It was convenient to choose for all these experiments a strain of *Esch. coli* which was strongly inhibited by deoxycholate citrate agar. It must therefore not be forgotten that both *Esch. coli* and other species vary greatly in the extent to which they are inhibited by deoxycholate citrate agar and other selective media. The media themselves also vary. The author has, on a number of occasions, found quite considerable variations between individual plates of single batches of medium poured at a single sitting and used at the same time for which, in spite of intensive search, no satisfactory explanation has been found. This variation was found both with deoxycholate citrate agar and with bismuth sulphite agar. Differing conditions of drying, in bulk dried plates of media, may have been responsible.

Several years ago (1954, 1955), Thomson drew attention to effects which may be very largely explained by the dominance of *Esch. coli* over salmonellae on solid media. Moore also (1950) and others have advocated measures which effectively limit the inoculum-density of *Esch. coli* on solid media, namely the use of diluted inocula for plating out.

SUMMARY AND COMMENT

This paper elaborates a concept of enrichment dynamics introduced in an earlier paper in which the author set out to find a labour saving technique for isolating salmonellae from sources other than faeces. Extensions to the concept are summarised in the Introduction. Explanations are given why a multiplicity of isolation procedures used in combination should normally yield more isolations than any one procedure alone.

Since a limit must always be set on the number of different media and procedures which can be used during routine examinations, a corresponding limit has also to be set on the combined efficiency of the procedures used. Not even a very laborious combination of methods can be expected to yield quite 100% of the isolations that are technically possible. When this rather unpalatable conclusion is accepted, a way is opened up for compromises between endeavour and reward, in terms of salmonella isolations. Judicious application of principles and methods discussed in these papers might be expected sometimes to lead to modifications in techniques which increase their efficiency, and in other cases to modifications which reduce labour without diminishing efficiency.

I take this opportunity of acknowledging help freely given me by members of the technical and clerical staffs of this laboratory.

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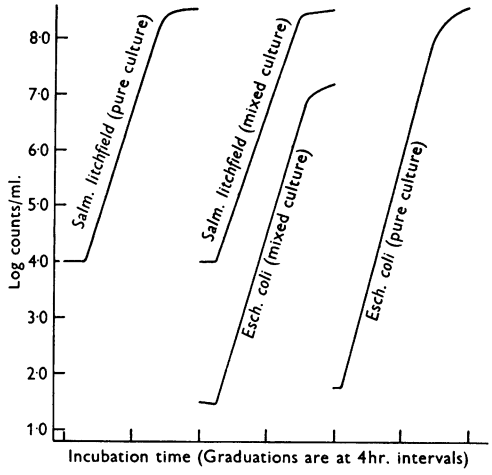

Fig. 1 Growth curves at 37° C., following unequal inocula to broth of *Salm. litchfield* and *Esch. coli*. together (centre). Also shown (left) is *Salm. litchfield* in sterile broth and (right) *Esch. coli* in sterile broth.

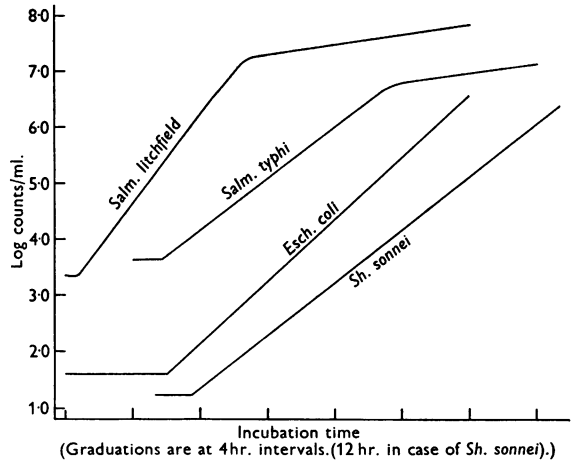

Fig. 2. Growth curves at 37° C. of various organisms, following inoculation to broths containing other organisms already at M-concentration. *Salm. litchfield* in *Esch. coli* (at M-conc.); *Salm. typhi* in *Esch. coli* (at M-conc.); *Esch. coli* in *Salm. litchfield* (at M-conc.); *Sh. sonnei* in *Esch. coli* (at M-conc.). The scale is the same as Fig. 1, except the time-scale for *Sh. sonnei*.

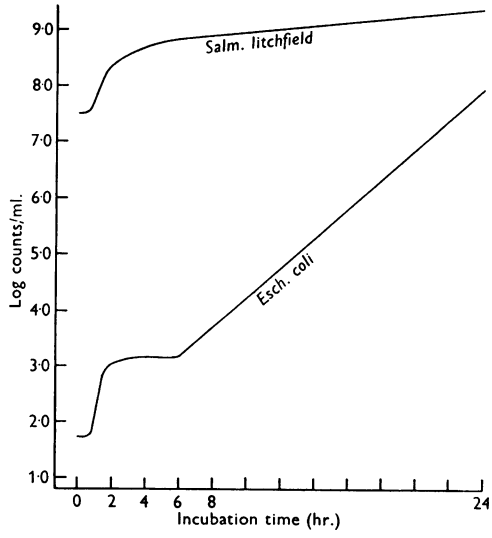

Fig. 3. Growth curves at 37° C., following very unequal inocula to broth, of *Salm. litchfield* and *Esch. coli* together, The time and number scale are the same as in Fig. 1.

APPENDIX

Co-ordinates of figs. 1, 2 *and* 3, *and media used for making the Miles & Misra counts*

	Time (hr.)	0	1	2	4	6	8	12	24	48	72	Medium
Fig. 1	Salm. (pure)	4·05	4·05	4·60	6·62	8·38	8·52	—	—	—	—	T
	Salm.	3·98	4·05	4·80	6·59	8·38	8·53	—	—	—	—	T
	Coli (mixed)	1·52	1·45	2·43	4·50	6·45	6·90	—	—	—	—	
	Coli (pure)	1·75	—	3·48	5·84	7·84	8·56	—	(8·90)	—	—	T
Fig. 2	*Salm. litchfield*	3·36	—	3·80	4·65	—	6·20	7·30	7·85	—	—	D-c
	Salm. typhi	3·65	—	3·70	4·15	—	5·06	5·97	7·15	—	—	D-c
	Esch. coli	1·60	—	1·56	1·62	—	2·14	3·18	6·58	—	—	T
	Sh. sonnei	1·23	—	—	—	—	—	—	2·60	4·36	6·40	D-c
Fig. 3	*Salm.* / *Coli* (mixed)	7·53 / 1·75	—	8·38 / 3·03	8·72 / 3·26	8·83 / 3·14	8·83 / 3·73	—	9·38 / 7·95	—	—	T

T, Teepol agar; D-c, Deoxycholate citrate agar.