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THE DECISIVE PERIOD IN THE PRIMARY INFECTION OF MUSCLE BY *ESCHERICHIA COLI*

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Summary.—*Escherichia coli* superinfected with a non-replicating phage was used to study the course of infection in the adductor muscle of mice in terms of the content of viable bacilli and the *in vivo* multiplication rate. The infection was characterized by a steady decrease of bacterial numbers to 5% of the inoculum within 4 hours without any compensating multiplication. Within the next 3 hours there was slight multiplication (2 generations or less) followed by a slow decrease of numbers to nil in 72 hours. In terms of the viable count, the infection was temporarily enhanced between three- and eight-fold when the following modifiers were given at the time of inoculation: local adrenaline, liquoid and ferric iron, systemic malonate and ferric iron, and hypovolaemic shock. Within 1–2 hours the inoculum was preserved from the bactericidal action of the muscle and multiplied to a limited extent (up to 3 generations). Given 2 hours after the inoculation, all the modifiers enhanced infection, but not when given 4 hours afterwards.

The results confirm the hypothesis, based on studies of local intracutaneous infections in the guinea-pig, that during the first few hours of infection, there is an extensive kill of the primary lodgement of bacteria by local defences that cease to operate after this period; and that the subsequent course of the local infection is determined by the number of bacteria surviving after this early decisive period.

THE course of a microbial infection may be described in terms either of some measurable reaction of the host or of the multiplication of the microbe *in vivo*. Both kinds of measurement are needed for a full understanding of the infective process.

Miles, Miles and Burke (1957) investigated the cutaneous inflammatory response of the guinea-pig to a variety of bacterial pathogens and deduced that the fate of the injected bacteria was decided during the first 3–4 hours of the local infection, and that at the end of this decisive period only a fraction of the inoculum, ranging from 10^{-1} to 10^{-6} according to the bacterial species used, had survived the local defences. Technical difficulties precluded routine direct counts of viable bacteria in the skin lesions, and their estimates of microbial survival were indirect, based on the linear relation between the diameter of the mature infective lesion and the log dose of living bacteria injected in a constant volume of suspending liquid. This paper describes experiments on the fate of *Escherichia coli* in infected mouse muscle, which, unlike skin, is readily homogenized for estimates of the

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content of viable bacteria. The experiments were made to test, in terms of bacterial numbers, the concept of the early decisive period and to explore the role of localization and dispersion in the suppression of the primary lodgement of the infecting microbe.

For a full characterization of the time course of multiplication, the number of living and dead bacteria in the lesion should be known. In practice, however, dead bacteria cannot be counted because some may have been destroyed and the remainder be difficult to recognize. The content of living bacteria is usually the only measure available. But this value is at any given moment the resultant of the rate of multiplication of the bacteria and the rate at which they are killed by the tissues. By itself, it cannot indicate the true multiplication rate.

However, as a result of Meynell's work (Meynell, 1959; Maw and Meynell, 1968), it is possible with *Escherichia coli* and *Salmonella typhimurium* to determine the true multiplication rate *in vivo*. In both instances, the strain of infecting bacterium carries a prophage and may be superinfected with a phage mutant of the prophage so chosen that, unlike the original prophage, it does not replicate with the bacterium. In a culture of multiplying superinfected bacteria, the mutant phage is progressively diluted out and over a given period the change of the ratio of superinfected viable cells to viable cells carrying only the prophage is a measure of the number of generations that have elapsed during that period.

Professor Meynell kindly gave us his strain of *E. coli* K12 (λh^+c^+), which carries the wild type prophage λb, from which he had produced the mutant superinfecting phage λhc. Ultraviolet irradiation releases both λb and λhc from superinfected cells so that plating an irradiated suspension on a strain of *E. coli* susceptible to both phages gives the total viable count, and on a strain of *E. coli* susceptible only to λhc, the number superinfected with λhc.

MATERIALS AND METHODS

Media.—Nutrient agar 1% (w/v) was used for colony counts by the method of Miles and Misra (1938) and tryptone agar 1% (w/v) as the base layer for plaque counts by the overlay method. Overlays were made with 2·5 ml of the tryptone medium in 0·6% (w/v) agar.

The *E. coli* strain K12 was grown overnight in nutrient broth and the indicator strains C600 and CR63 for 4 hours, with aeration, in tryptone broth.

Phosphate buffer for dilutions of bacterial suspensions was prepared as follows: 4 g NaCl, 5 g K_2SO_4, and 1·5 g KH_2PO_4 were made up in 1000 ml distilled water; after sterilization, 1·0 ml of 0·1 M/l $CaCl_2$ and 1·0 ml of M/l $MgSO_4$ were added; and for diluting phage suspensions, 5 ml of 0·04% gelatin was added to each 200 ml of the buffer.

Bacteria.—Stocks of *E. coli* strain K12 (λh^+c^+) and the indicator strains C600 and CR63 were maintained on Dorset's egg slopes at 4°. C600 was suceptible to the lysogenic wild type phage λb and to the mutant λhc, and CR63 susceptible only to λhc. Nutrient agar plates bearing discrete colonies were prepared for daily use and kept at 4°. After overnight incubation, cultures contained about 8×10^8–2×10^9 viable organisms/ml.

Phage.—Stocks of phage λhc were made as follows: Ten-fold dilutions of a λhc suspension were added to tryptone broths containing thiamine, magnesium sulphate and *E. coli* strain C600 in the late log phase of growth. Incubation at 37° resulted in rapid clearing after 3–6 hours. Centrifugation and filtration of the most rapidly lysed suspensions through Seitz-type filters previously primed with tryptone broth yielded bacteria-free stocks of λhc, containing 10^9–10^{10} plaque-forming particles/ml.

Ultraviolet irradiation.—Before irradiation, the suspensions of bacteria were diluted 1/100 in phosphate buffer. Samples of 5 ml were placed 52 cm below a 20 watt discharge ultraviolet lamp and irradiated on a rocker for 40 seconds at room temperature. From 99·00 to 99·99% of superinfected *E. coli* strain K12 (λh^+c^+, λhc) were lysed by this method. After irradiation, the organisms were incubated at 37° for 55 minutes in the dark to prevent reversal

of the irradiation effect. The activated suspensions, either cultures or muscle homogenates, were titrated on strains C600 and CR63.

Superinfection.—Since stationary-phase *E. coli* adsorbs phage optimally, overnight cultures at 37° were used. Mixtures of λhc and strain K12 at a multiplicity of one were incubated at 37° for 15 minutes. From 99·00 to 99·99% of the superinfecting phage was adsorbed under this condition. The mean number of phage particles adsorbed per bacterium could therefore be assumed to be equal to the ratio of the initial concentration of phage to the initial concentration of bacteria. The numbers of viable bacteria and the titre of unadsorbed phage were determined in every experiment.

In vitro *check of the superinfection technique.*—The accuracy of the superinfection technique was from time to time checked with cultures *in vitro*, by comparing generation rates derived from direct viable counts with rates derived from the λhc/λb ratios. As the representative test in Table I shows, the number of generations estimated by the 2 methods differed by less than unity.

TABLE I.—*Growth of* E. coli *in Broth, Estimated from Viable Count and Dilution of Non-replicating Bacteriophage*

	Viable count		Superinfection method	
Age of culture (hours)	Number/ml	Cumulative number of generations	λhc/λb	Cumulative number of generations
0	$7·1 \times 10^4$	0·0	0·29	0·0
1	$1·4 \times 10^7$	1·0	0·22	0·0
2	$2·0 \times 10^6$	4·9	0·017	4·2
3	$1·7 \times 10^7$	7·9	0·0023	7·0
4	$9·5 \times 10^7$	10·3	0·00015	10·9

Animal inoculation.—Suspensions of the strain K12 ($\lambda h^+ c^+$) were injected from a microsyringe into the adductor muscle of mice in volumes of 0·005 to 0·010 ml, containing from $4·5 \times 10^3$ to $8·5 \times 10^7$ bacteria. After a prescribed interval, the animals were killed by cervical dislocation and the entire adductor mass was excised with aseptic precautions; the muscle was minced, placed in 5 ml of chilled saline and homogenized with a Teflon piston for 5 minutes. Viable counts were made directly on to nutrient agar.

When *in vivo* growth rates were to be determined from the λhc/λb ratios, both the inoculum and the muscle homogenate were irradiated, and 0·1 ml of appropriate dilutions were plated with 0·25 ml of the suspension of the indicator strain C600 and CR63 and 2·5 ml of soft agar.

The bacterial growth and death rates are calculated as in the following example. If 10^7 *E. coli*, with a ratio λhc/λb of 0·50 (*i.e.* half of them superinfected), are inoculated and after 3 hours 10^6 are recovered, with a λhc/λb ratio of 0·031, clearly 4 generations (0·50 → 0·25 → 0·12 → 0·062 → 0·031) have elapsed and the 10^6 living organisms represent the growth from $10^6/2^4 = 6·2 \times 10^4$. The kill during this period is $10^2(10^7 - 6·2 \times 10^4)/10^7 = 99·38\%$, and the mean generation time is 45 minutes.

Mice.—Two strains (CFW and SAS) of white mice weighing 20–40 g were used. The results with both were similar.

RESULTS

Immediate recovery of injected bacteria

In normal mice killed immediately after inoculation, the average proportion of bacteria recovered was 44%. Ninety-two % of all the estimates fell between 22 and 72%. The proportion of bacteria recovered was approximately linear with respect to the log dose of bacteria injected. From Fig. 1 it will will be seen that with doses from 10^5 to 10^8, a 40–80% recovery is to be expected. The better recovery with the higher dose is probably an expression of the tendency of injected particles, like bacteria, to accumulate in the tissues immediately round the needle point, more so with thicker suspensions than thinner. The recovery of bacilli from large clusters at the centre, where many are not directly adsorbed to the

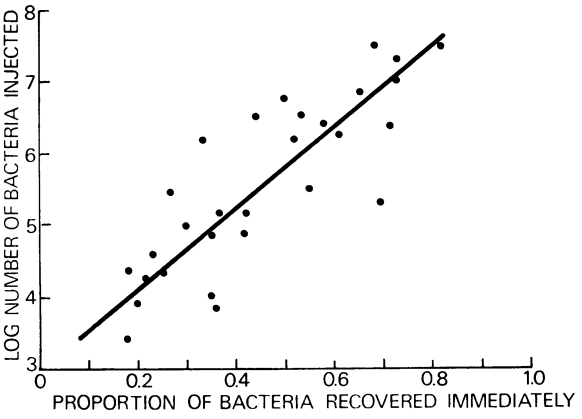

FIG. 1.—Recovery of viable *E. coli* immediately after inoculation of muscle.

tissues, is therefore likely to be better than from more distant bacilli lodged singly or in small clusters. The failure to recover all the injected organisms may have been due either to destruction of bacteria during homogenization or to rapid killing on contact with living mouse muscle. But recovery was similar (31, 49 and 59%) in animals killed immediately *before* and sampled immediately after inoculation and (22, 44 and 79%) in animals killed and maintained at 37° for 4 hours before inoculation and sampled immediately after. Furthermore, the muscle homogenate itself was not bactericidal over a period of 4 hours at 37°, indicating that a continued bactericidal action between killing the mouse and plating the homogenate is unlikely.

However, undiluted mouse serum prevented growth of the test bacteria during 1 hour at 37°; in the same conditions 1/10 serum retarded growth after 1 hour, 1/100 after 4 hours, and 1/100 had no effect during 4 hours. Since the concentration of serum proteins in muscle homogenate is unlikely to be greater than 1/100, the bactericidal effect of the serum is unlikely to be operative in the short period between excision of the muscle and plating of the homogenate.

An alternative reason for the poor recovery is dispersion of the injected bacteria to sites outside the adductor mass. This alternative was tested in 2 ways. First, ileo-inguinal lymph nodes were excised, homogenized and cultured at intervals after muscle inoculation. They all proved to be sterile throughout a 24-hour period. Second, the usual excision and homogenization of the adductor mass were carried out, and the rest of the hindquarter was then amputated, homogenized and cultured. In 10 of 13 animals studied over a course of 4 hours less than 1% of the bacteria recovered from muscle was present in the remainder of the hindquarter. Of the remaining 3, in only one was the value greater than 3%. Bearing in mind the possibility that the tissues outside the adductor mass were contaminated during its excision, dispersion into adjacent tissues or regional lymph nodes appears to contribute little to the failure to recover from the muscle all the bacteria injected.

It appears that as a rule incomplete dispersion of clumps of injected bacteria, and perhaps some killing of bacteria during homogenization is responsible for the mean recovery rate of 44% immediately after inoculation.

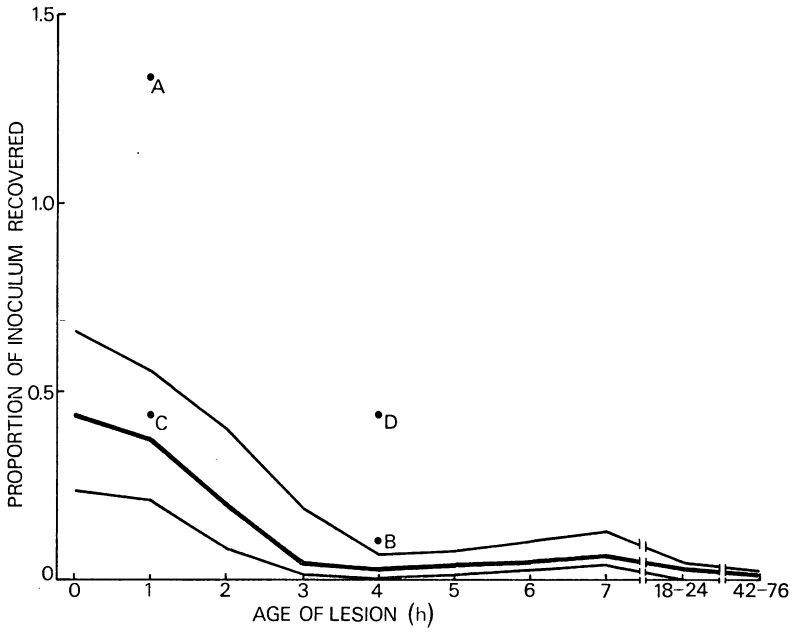

FIG. 2.—Recovery of viable *E. coli* from infected muscle. The mean values (thick line) and upper and lower observed limits are plotted. A–D, see text p. 105.

Surival of E. coli *in the muscle of normal mice*

The change with time of the mean bacterial content of muscle inoculated with doses of 10^4–10^7 living *E. coli* is shown in Fig. 2. It is based on the examination of 203 mice, at least 18 separate estimates being made for each point on the curve.

Bacterial growth, as indicated by the λhc/λb ratio (Table II), did not occur during the first 3 hours. There was variable growth, equivalent to 2 generations or less, during the fourth to the seventh hour, and no growth thereafter. The transient increase (Fig. 2) in living muscle at the seventh hour was consistently observed, as was the subsequent decline in numbers and the survival of a few bacteria for up to 7, but not more than 7 days.

TABLE II.—*Percentage (P) of Inoculum of Superinfected* E. coli *Recovered from the Adductor Muscle of Normal Mice, and Ratio (R = λhc/λb) of Superinfected to Non-infected Bacilli*

Hours after inoculation	Number of viable *E. coli* injected: $3{\cdot}0\times10^5$ P	$3{\cdot}0\times10^5$ R	$4{\cdot}0\times10^6$ P	$4{\cdot}0\times10^6$ R	$1{\cdot}2\times10^7$ P	$1{\cdot}2\times10^7$ R
0	26	—	35	—	44	—
1	28	0·315	33	0·216	42	0·417
2	20	0·361	18	nt	23	0·445
3	6	0·390	7	0·260	10	nt
4	5	0·334	4	0·227	5	0·460
7	12	0·168	6	0·194	11	0·218

nt = not tested.

The scatter of values round the mean for each point on the curve is relatively small. Consequently, in tests with systemic modifiers no simultaneous control tests were made as a rule, the mean " control " curve being used as a basis for comparison. Tests in untreated animals were nevertheless made from time to time to establish a continuing uniformity of response in the mouse population used. With local modifiers, their effect in each animal was compared directly with the outcome of a control infection in the opposite adductor muscle.

The early decline in the viable count is evidently due to active killing by the living muscle, because when mice were killed, immediately inoculated and held at 37°, microbial growth was unimpeded, as evidenced by the similarity of the number of generations estimated from the viable count and by the superinfection technique (Table III).

TABLE III.—*Growth of* E. coli *at 37° in Adductor Muscle of Dead Mice, Estimated from the Viable Count and Dilution of Non-replicating Bacteriophage*

	Viable count		Superinfection method	
Hours after inoculation	Number per adductor muscle	Cumulative number of generations	$\lambda hc/\lambda b$	Cumulative number of generations
0	$1{\cdot}5\times10^{6}$	0·0	0·30	0·0
2	$4{\cdot}0\times10^{6}$	1·6	0·15	1·0
3	$6{\cdot}1\times10^{6}$	2·0	0·082	1·9
4	$1{\cdot}4\times10^{7}$	3·1	0·071	2·2
6	$2{\cdot}5\times10^{7}$	4·0	0·032	3·3
7	$6{\cdot}0\times10^{8}$	8·7	0·0015	7·7

It is clear that in the first 4 hours the body defences kill some 97% of the inoculum. The survivors are capable of a little further growth, but not to the extent of establishing either a local or general progressive infection. Clearly, in this site the K12 strain is a poor pathogen. An intraperitoneal dose of 10^{9} viable bacteria killed the mice in 18 hours, but the death appeared to be due to intoxication, since by the superinfection method no intraperitoneal growth was detectable during this period.

Although, as already noted, the percentage initial recovery of inoculated bacteria increases with increasing inoculum, the individual time course of bacterial numbers was the same with inocula differing up to a thousand-fold, justifying the use of an inoculum of between 10^{5} and 10^{7} for comparative tests. The inocula used ranged from 8×10^{5} to 4×10^{7}; in 95% of the tests they ranged from 10^{6} to 9×10^{6}.

Survival of E. coli *in the muscle of treated mice*

The action of a number of modifying procedures or agents known to enhance infections was tested in the muscle—*E. coli* system, namely hypovolaemic shock, and local adrenaline and liquoid (Miles *et al.*, 1957), systemic malonate (Berry and Mitchell, 1953) and both local and systemic ferric iron (see Weinberg, 1966, 1971). All these agents enhanced the *E. coli* infection to some extent.

Calculation of the enhancement factor.—A simple, sufficiently informative numerical expression of the factor of enhancement in terms of the number of viable bacilli in the lesion is difficult to devise. Polk and Miles (1971) expressed it as the ratio of recovery rates in treated and control muscle at the time when the difference in counts was maximum. Thus (Fig. 2) if the maximum was at

1 hour a recovery rate of 1·31 (point A), when the mean control rate is 0·375, indicates an enhancement factor of 3·5. But at 4 hours with a control rate of 0·031, a 3·5-fold enhancement would follow from a low recovery rate of 0·108 (point B). Again, with a recovery rate of 0·44 at 1 hour (point C) resulting from simple survival of the inoculum, the factor would be 0·44/0·375 = 1·1; but with the same recovery at 4 hours (point D) it would be 0·44/0·031 = 14·2. To avoid such exaggerated estimates when the maximum bacterial content in enhanced lesions occurs in the third to the sixth hour, we have in this paper used as the starting point of the enhancement the control count at the time the modifier was given; *i.e.*, the population of viable bacteria upon which the modified tissues will act. For modifiers given at 0 hour the control count, expressed as recovery rate, is 0·44; at 2 hours 0·20 and at 4 hours 0·031. Thus, in Fig. 3 the enhancement factor for adrenaline given at 0 hour is 2·60/0·44 = 5·7 and for adrenaline at 2 hours it is 1·10/0·20 = 5·05.

Hypovolaemic shock.—Shock was induced by the intraperitoneal injection of 50% dextrose in water, given immediately before the intramuscular inoculation. When the dextrose killed the animal it did so within 6 hours. Measured at this time the percentage lethality of the solution, in ml/g body weight, was as follows: 0·22 ml, 0; 0·33 ml, 20; 0·03 ml, 80; and 0·044 ml, 100. Accordingly, the approximate LD_{20}, 0·033 ml/g, was used. This dose, however, did not regularly induce shock. Enhancement of infection was also irregular but averaged about four-fold. It had no relation to the degree of shock since the higher degrees of enhancement occurred in slightly ill, sick and apparently healthy mice. Bacterial division, when detectable, took place sluggishly. When shock was induced 1 hour before inoculation, the enhancement was maximal 1 hour sooner and of the same order of magnitude.

Adrenaline.—Adrenaline was used in a locally vasoconstrictive dose. The minimum effective dose was that amount in 0·01 or 0·005 ml volume which, when injected intramuscularly with 0·5% methylene blue, led to a decolourization of the methylene blue within 30 minutes (Evans, Miles and Niven, 1948). Two μg of adrenaline proved to be effective. Given with the bacilli it enhanced maximally between the first and second hour, as exemplified in Fig. 3. Sometimes the maximum occurred at the second hour but a 1-hour maximum could be assured by giving the adrenaline 1 hour before the bacilli. From the λhc/λb ratios it is evident that the bacilli grew during the period of enhancement, but by the third and fourth hour the defences are once more active, and the subsequent course of infection was like that in untreated muscle. Enhancement ranged from three- to eight-fold.

Liquoid.—Liquoid (sodium polyanethol sulphonate) is anticoagulant, anti-complementary and antiphagocytic. Injected simultaneously with *E. coli*, 50–100 μg induced a three- to six-fold enhancement from the second to the fifth hours (and three-fold at the third hour in Fig. 4). As with adrenaline-treated muscle, there was a rapid decline in numbers to control values by the seventh hour. There was relatively rapid bacterial growth during the second and third hour.

Ferric ammonium citrate.—Iron in this form in doses of 8–16 μg ferric ions, given either intramuscularly or intraperitoneally, enhanced infection from three- to six-fold. The maxima tended to occur about the fifth hour after local and about the third hour after systemic Fe^{+++} (Fig. 5).

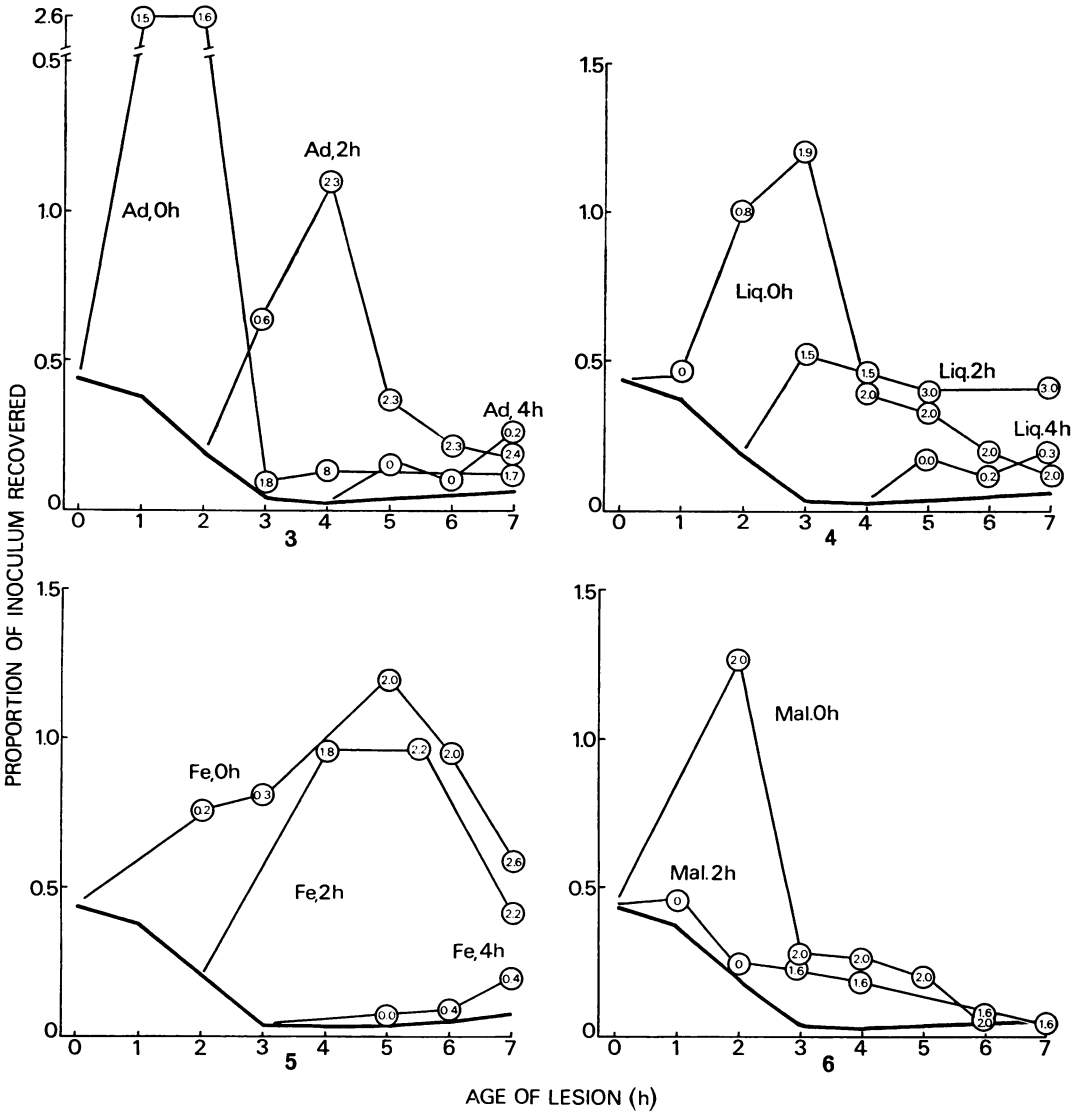

FIG. 3–6.—Recovery of viable *E. coli* from infected muscle of mice treated with various modifiers, compared with mean recovery rates in normal mice (thick line). The figure in the circles is the cumulative number of bacterial generations at the time of sampling. The modifiers were given at the time of inoculation (0 hour) and 2 and 4 hours after. Fig. 3.—Local adrenaline, 2 μg. Fig. 4.—Local Liquoid, 50–100 μg. Fig. 5.—Local Fe^{++}, 8–16 μg. Fig. 6.—Systemic malonate (for dosage see text, p. 107).

Bacterial growth was contemporaneous with the enhancement, but occurred at rates less than those with liquoid.

Malonate.—Berry and Mitchell (1953) showed that systemic disodium malonate, a competitive inhibitor of succinic oxidation, lowered resistance to *Salmonella* infection in mice.

Intraperitoneal doses of 20 mg of sodium malonate in 0·2 ml of saline at 0, 1, 2, 3, 4, 5, 6 and 7 hours more effectively enhanced the *E. coli* than a single dose of 50 mg in 0·5 ml at 0 hour, which was the maximum sublethal dose. Three- to four-fold enhancement occurred after 3–4 hours, but not earlier (Fig. 6). The $\lambda hc/\lambda b$ ratios indicated about 2 generations of bacterial growth.

It is evident that all the modifiers arrest the death of the bacilli in the muscle and allow a limited multiplication. In the doses used, however, their effect is transient since in all instances multiplication ceases after 3–5 hours and killing again begins, so that, as in untreated muscle, most of the bacilli are killed within the next few hours.

The tissue reaction to this infection was slight. In neither untreated nor treated muscle was there any naked-eye sign of hyperaemia, infiltration or necrosis during the first 7 hours infection, or at 18–24 or 72 hours. Microscopically, a moderate tissue leucocytosis began about the third hour, was maximum during the next hour or so, and gradually declined over the next 24 hours.

It is not surprising that the tissue reaction to the enhanced infection should be no more severe than those in untreated muscle. The inoculum was an 18-hour culture, in which it may be assumed that of every 100 bacilli at most only 10 are viable and 90 are dead. At maximum enhancement, 3 generations of bacilli have occurred, and the 10 have become 80. After 5 hours, when most of the bacilli are dead, there will be $90 + 80 = 170$ bacillary bodies in the enhanced lesions for every 100 in control lesions. It is unlikely that an increase of the order of 1·7-fold in the number of dead bacilli would be detectable as an increased inflammatory response of the muscle.

The decisive period

Miles and his colleagues (1958) observed that intracutaneous infection in the guinea-pig by a number of pathogens, including *E. coli*, was enhanced when local or systemic modifiers were applied at the time of infection. The enhancement was evident from the size of mature 20-hour lesions. When the modifiers were applied in animals bearing 3–4 hour old lesions, the mature lesions at 20 hours were no larger than those in untreated skin. They concluded that the local defences inhibited by the modifiers were decisive in determining the outcome of the infection and, since they were not affected by modifiers given at 3–4 hours, that they were operative only during that short decisive period. The results with *E. coli* in mouse muscle confirm that conclusion. All 5 modifiers when applied to mice bearing lesions established for periods from 1 to 4 hours were ineffective at 4 hours. Some of the results are exemplified in Fig. 3–6. Given at 2 hours adrenaline, liquoid and ferric iron enhance 5·5-, 2·6- and 4·8-fold respectively, as against 7·0-, 2·7- and 2·7-fold when given at 0 hour. Malonate given hourly from the second hour of infection was much less effective than when given hourly from the time of infection. None of the modifiers enhanced when given at 4 hours. The slight increases in count observed from the fourth to the seventh hour are well within normal limits (Fig. 2) and the estimated numbers of generations by the seventh hour, 0·2–0·4, is less than the 1·5–2·0 (Table II) observed in control infections.

The tests with each modifier were repeated several times, with similar results. In summary, the time of maximum enhancement with simultaneous modifier

and inoculation was 1–2 hours with systemic iron, 2–3 hours with local adrenaline, liquoid and iron, 2–3 hours with systemic malonate, and 3–4 hours with shock. The factor of enhancement varied from 3 to 8, and multiplication for 1–3 generations (usually 2) took place. The decisive period, after which modifiers were ineffective, was 2–3 hours with iron, 3 hours with malonate, and 2–4 hours with adrenaline and shock.

DISCUSSION

The enhancement of the feeble *E. coli* infection in mouse muscle corresponds with that caused by shock, liquoid and adrenaline in more substantial intracutaneous infections, including infection by *E. coli*, in the guinea-pig (Miles *et al.*, 1958); by malonate in systemic *Salmonella* infection of the mouse (Berry and Mitchell, 1953); and by ferric ions in systemic *E. coli* infections in the guinea-pig (Bullen, Leigh and Rogers, 1968).

Our indicating reaction was the content of viable bacilli in the infected adductor muscle. The measurement also, by the phage superinfection technique, of the rate of bacterial multiplication makes it clear that the gradual killing of the inoculum during the first 4 hours is not mitigated by any multiplication of some of the bacilli. After 4 hours, some 3% survive and multiply slowly until the seventh hour, after which their numbers again decline. This 7-hour peak was the only obvious manifestation of the pathogenicity of the strain of *E. coli* used. Its early death, and its subsequent phase of growth, correspond to the early kill and subsequent maturing of the infective lesion postulated by Miles, Burke and Miles (1957) for skin infections in the guinea-pig.

When applied at the time of inoculation, all the enhancing agents tested appeared to check the early killing of the bacilli immediately, preserving them so that at least some could multiply—in some cases during the next hour, in others (*e.g.* Fig. 6) an hour or so later. This preservative action was even more evident in a few of our tests. For example, with adrenaline (Fig. 3) the recovery rate rose in 1 hour from 0·44 to 2·6, a seven-fold increase. This corresponds to 3 bacterial generations but rather less than 2 were observed. Technical errors apart, this discrepancy is resolved only by assuming that 100% of viable inoculum, instead of the usual 44%, had been preserved from the moment of injection.

The results of Miles and his colleagues with the late administration of modifiers were also confirmed. None had any effect when given to animals bearing lesions aged 3–4 hours. For infected mouse muscle, as for guinea-pig skin, we postulate accordingly that the defences inhibited by the modifiers operate during a decisive period of a few hours, after which the modifiers are ineffective because the defences they inhibit cease to operate. In muscle, the outcome of the infection so decided is the 7-hour peak of bacterial multiplication; in the skin it was the size of the mature lesion after some 20 hours. Shock and adrenaline act presumably by diminishing the supply of bloodborne antibacterial agents to the muscle; liquoid because it is anticomplementary. Malonate appears to poison the tissue defences by inhibiting the oxidative tricarboxylic acid cycle, with a consequent accumulation of unoxidized metabolites (Berry and Beuzeville, 1960).

The dose of systemic iron was sufficient to saturate iron-binding proteins like plasma transferrin, with which in the normal animal the bacteria have presumably to compete for their iron and to provide free ferric ions for the *in vivo* growth of

the inoculum (Bullen *et al.*, 1968; see also Fletcher and Goldstein, 1970; Weinberg, 1971); a similar explanation probably holds for the action of local iron.

E. coli was chosen for our experiments partly as an exemplar of the feeble pathogens which infect tissues traumatized by accident or by surgery but also because it was one of 2 organisms so far available—the other being a strain of *Salm. typhimurium*—of which it is possible to measure the division rate *in vivo* by the use of a superinfecting, non-replicating phage. With known division rates it is possible to determine to some extent to which the viable count *in vivo* at a given moment is the result of the death of some and the multiplication of others of the infecting microbes. The analysis is highly informative with a progressive salmonella infection lasting some days (Maw and Meynell, 1968). With our *E. coli* infection, where an episode of killing was followed by a short burst of growth, division rates served mainly to confirm the results obtained by viable counts. Nevertheless, they established that with this organism the early period of kill was unremitting, and confirmed the multiplication indicated by the rise in count at 7 hours which, in the light of the error of our estimates during this period of the infection, was only dubiously established by viable count alone (Table II).

We are greatly indebted to Professor G. G. Meynell for his help with the phage superinfection technique.

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