VIRULENCE AND IMMUNIZING CAPACITY OF SALMONELLA TYPHIMURIUM AS RELATED TO MUTATIONS IN METABOLIC REQUIREMENTS[1]

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Received April 22, 1953

ENVIRONMENTAL and genetic influences acting on and within pathogen and host have been shown to be important in resistance to disease. The level of mortality and morbidity of infectious typhoid in domestic fowl or mice as shown by the many years of research at Iowa State College is dependent upon the proper relations between the genetic constitutions of the pathogen and of the host. In a sense, the severity of a disease may be represented as a surface of a solid model. The genetic constitutions of the hosts are on one side of the base. At one end specified doses of the pathogen cause complete mortality to hosts of the highly susceptible constitution. In the middle, like doses induce 50% deaths. At the other end, similar doses scarcely affect the activities of the hosts (GOWEN 1948, 1952).

The genetically different typhoid pathogens are on the other side of the square. At one end, a genetic line of the bacteria is practically innocuous to any strain of the host. In the middle, the genotypes of the lines endow them with capacities to kill most of the susceptible mice, allow the survival of about 50 percent of the medium susceptible mice, and nearly all of the resistant mice. At the other end, the virulence of the pathogen line is so great that some mice of even the resistant strain die when inoculated with the standard dose of organisms.

Over the surface of the square the different genetic constitutions of host and pathogen fit together in such a manner as to give all types of disease reactions. This key to lock mechanism suggests the next steps if disease is to be understood. Information is needed on what the genes do to modify the different lines of the pathogen and thus induce the different disease reactions. A growing body of evidence indicates that a gene causes its primary reaction by contributing to the synthesis of a required chemical at some particular step in its formation. For a pathogen some of these steps may be significant to the organism's invasive ability as in the release of toxic metabolites, endotoxins, and in competition for host metabolites, etc.

[1] Journal Paper No. J-2109 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project Nos. 1180 and 1187. This work has received assistance from Contract No. AT(11-1)107 from the Atomic Energy Commission.

To test these questions and to search for lines of pathogens which may be utilized in further virulence studies, mutants unable to synthesize certain essential growth elements were isolated from three pure cultures of *Salmonella typhimurium* over a period 1946 to 1950. The first culture, 519, originally came from a human food poisoning case in New York. The second and third cultures, 533 and 533-11C, were transfers from our line 11C, under study in our laboratory for some 25 years. The mutants were obtained as isolates from X-ray or ultraviolet treated cultures. Mutants are specified by number and the growth substance they have come to require at the time of mutation. These isolations were made at Amherst College by H. H. PLOUGH and HELEN MILLER. The techniques of initiation and separation of these mutants may be found in the literature cited (PLOUGH, YOUNG and GRIMM 1950; PLOUGH, MILLER and BERRY 1951).

The studies of natural resistance and acquired immunity were conducted at Iowa State College by J. W. GOWEN and J. STADLER, with the assistance of DOUGLAS GRAHN and PATRICIA BANACH, during 1948 to 1951.

TABLE 1

Differences between experiments within strains of mice. Tests with S. typhimurium line 11C.

Strain of mice	Number tested	Percent survived	Between experiments within strains		
			χ^2	d.f.	P
Balb/Gw = Ba in text	120	0	0	4	1.0
LGW = L in text	33	33	2.4	3	.5
Z	35	77	8.0	3	.05
S	29	100	0	2	1.0

Sixty-seven independent *S. typhimurium* mutants and their four parent lines were randomly selected for this study. Twelve mutants came from culture 519, 30 from 533 and 25 from 533-11C. These mutants were tested for their virulence on four of our inbred strains of mice. 200,000 organisms per mouse were injected intraperitoneally in each mouse tested. The results of these tests measure the virulence of the mutant bacterial line.

After recovery from the experimental typhoid infection, mice acquire some active immunity. The extent of this immunity was assayed by inoculating a challenge dose of 50 million virulent 11C organisms. The results of this test measure the power of the mutant bacterial line to stimulate active immunity in the host. Certain factors, significant to the adequacy of the tests, as strain and sex of host, average virulence or immunizing power of the mutants, repeatability of tests, etc., will be examined before considering the mutants individually.

Repeatability of virulence tests

Five separate experiments were conducted. Four inbred strains of mice served as the test hosts. Mouse typhoid culture line 11C was used as a control in each of the experiments. Table 1 shows how repeatable these tests were.

TABLE 2

Sex differences in resistance—First test. Strain Ba.

Experiment	Total mice tested	Percent survived		χ^2	P
		Males	Females		
I & II	475	85.6	90.8	2.9	.10
III	463	66.1	60.1	1.9	.15
IV	472	23.1	21.9	.1	.6
V	1092	80.4	83.1	1.6	.2
Total	2502	68.2	68.5	.7	.4

The P values for the different strains indicate that the different experiments furnish similar information.

The percent survival for the different mouse strains shows the host effects range from the highly susceptible Ba mice to the highly resistant S mice. The L and Z strains were somewhat more resistant than expected from the large accumulated body of data available from past work.

The resistance of the S mice is such that they will withstand attacks of almost any line of *Salmonella typhimurium*. This 100 percent survival does not allow differentiation of virulence in the various mutants. Similar considerations apply to the Z mice and to a lesser extent to the L mice. The Ba mice contribute the greatest amount of information so these data are chosen for presentation.

The data of the different experiments within strains of mice were comparable so they were combined for further analysis.

Sex effects on resistance

In general the individual tests of the different mutants were balanced as to sex of the host. Table 2 gives the survival value for the males and females in each experiment together with the χ^2 tests for the homogeneity of the results.

The data on Ba mice of table 2 show that the males and females react in like manner to their initial contact with the mouse typhoid organisms, probabilities being such as easily obtained through chance difference.

Table 3 gives similar data on the challenge tests for active immunity.

TABLE 3

Sex differences in resistance—Challenge test. Strain Ba.

Experiment	Total mice tested	Percent survived		χ^2	P
		Males	Females		
I & II	394	58.9	65.4	1.8	.19
III	265	64.8	54.0	3.2	.07
IV	100	70.4	58.7	1.5	.22
V	804	45.0	40.6	1.6	.20
Total	1563	53.4	50.5	1.2	.27

These data indicate that the sexes become actively immunized to the typhoid organisms of the different lines to a like extent. The data for the sexes are combined in the rest of the study.

Comparison of host strain survivals for all mouse typhoid lines

The inbred mouse strains utilized in these tests exhibit established differences in resistance. The differences as tested by a challenge dose with the virulent 11C parent culture were shown in table 1. The differences when the mice are challenged by the mutant *S. typhimurium* lines are shown in table 4.

The host strain differences in resistance are evident in table 4 where all of the different mutant strains are grouped together. The survivals of all strains on first contact with the disease are greater than those observed when these mice are exposed to the parent strain *S. typhimurium* 11C. As would be expected these differences in susceptibility become more evident as the tests progress from the resistant toward the more susceptible hosts. On the average, mutation of a virulent bacterium tends to reduce the virulence of the parent bacterial lines.

TABLE 4

Survival of mice where the mutant S. typhimurium lines initiated the disease.

Strain	First test			Challenge test		
	Number tested	Number survived	Percent survived	Number tested	Number survived	Percent survived
Ba	2262	1649	73	1563	803	51
L	807	653	81	553	373	67
Z	667	580	87	573	482	84
S	497	488	98	440	412	94

The progressively increasing proportion of survivals from the susceptible Ba mice to the resistant S show that the acquired immunities as well as the natural resistances are under genetic control of the host. Our earlier immunization experiments utilizing line 11C and two of its derivatives led to like conclusions (GOWEN 1937–1947).

The immunity acquired by the mice from the mutant *S. typhimurium* lines, table 4, is generally less than the immunity acquired when the parent culture 533-11C offers the initial challenge (100 percent survival for all hosts in these experiments). Mutation, on the average, has lowered the immunizing ability of the mutant line.

The general reduction in virulence of the mutants tends to reduce the differences in the outcome of the tests for virulence as the mouse strains employed in the tests are taken from stocks of higher natural resistance. In other words the data from the experiments on the Ba mice are most significant for separating the mutant effects on virulence and immunizing against mouse typhoid. The experiments employing the L, Z and S are respectively less and less significant. For this reason most of the subsequent analyses will deal with the

TABLE 5

Relation between natural resistance or acquired immunity of Ba and L mice tested with different S. typhimurium mutants.

Constants	Natural resistance	Acquired immunity
Mean resistance of Ba, % survival	65	65
Mean resistance of L, % survival	91	56
Correlation coefficient between Ba and L resistance	0.61**	0.88**

*Shows significance in the 0.05–0.01 probability range.
**Beyond 0.01 range for this and subsequent tables.

results of experiments in which the Ba strain of mice was employed and omit those for the other host strains.

Before leaving this point, however, data are presented below to show that, when tested in the same experiments, the results secured with Ba mice have a high correlation with results on the L mice in both the response to first contact with the disease and in the acquired immunity tests.

The different strains of mice are known to have certain elements in common as well as certain differences which determine their respective resistance to *S. typhimurium*. The correlations of table 5 measure these effects. The Ba and L mice show quite similar reactions even though the L mice are distinctly more resistant than the Ba strain. It appears probable that if the dosages of test organisms and other conditions were adjusted properly, the Z and S strains of mice would show common pathways of resistance to the different *S. typhimurium* mutants comparable with those observed for the Ba and L mice as well as unlike mechanism of resistance having importance to the given strain. As the experiments were conducted, the spread in survival rates is greater for the Ba mice than for those of any of the other strains. The Ba mice consequently give a clearer differentiation for separating the effects of mutations on virulence and acquired immunity. This strain is used for the subsequent evaluations of the mutants.

TABLE 6

Variance of bacterial mutants in virulence—First tests. Ba mice. Bacterial lines.

Source of variance	d.f.		Sum of squares		M.S.	
	All data	Only those with repeat tests	All data	Only those with repeat tests	All data	Only those with repeat tests
Among bacterial lines	66	36	313.3	98.1	4.75**	2.72**
Within bacterial lines among tests	42	42	29.5	29.5	.69**	.70**
Within bacterial lines and tests	2152	1106	104.1	72.0	.05	.07
Total	2262	1184	446.9	199.6		

Stability of virulence and immunizing properties of the S. typhimurium mutants

The metabolite mutants were repeatedly tested within the different experiments. These experiments give a measure of the consistency with which the different mutants maintain their virulence. The data are shown in table 6.

The variance analysis of the binomial data, table 6, shows that the *S. typhimurium* mutants are different from each other in their virulence.

The experiments themselves were shown to be homogeneous when tested with the 11C culture, P = 0.5 to 1.0 for the four host strains, data not shown. The test difference within bacterial lines shown may be attributed to some balancing interaction between local conditions within the experiment.

A similar analysis for the stability of the immunizing properties of the mutants is shown in table 7. The different *S. typhimurium* mutants are shown to be significantly differentiated from each other in their abilities to stimulate acquired resistance in the host. These mutants display some differences in their abilities to immunize in different experiments.

TABLE 7

Variance of bacterial mutants in virulence—Challenge tests. Ba mice. Bacterial lines.

Source of variance	d.f.		Sum of squares		M.S.	
	All data	Only those with repeat tests	All data	Only those with repeat tests	All data	Only those with repeat tests
Among bacterial lines	54	35	218.9	101.1	4.05**	3.0**
Within bacterial lines among tests	40	40	26.3	25.9	.65**	.65**
Within bacterial lines and tests	1411	784	131.2	87.0	.09	.11
Total	1505	857	376.4	214.1		

Effect of metabolite requirement on pathogenic properties of S. typhimurium mutants

The bacterial mutant lines were of several types. Thirty-nine of the 67 mutants require but a single known metabolite for growth. Fourteen were shown to require one of two different metabolites. Of this number six lines were composed of two different and separable bacterial types, one requiring one metabolite and the second type requiring the other metabolite. Presumably these six lines arose in the original culture as separately mutated bacteria which stuck together on transfer, or as two mutations in different nuclei of a multinucleate bacterium. In the latter case the two nuclei would subsequently separate by division and become incorporated in independent organisms as a consequence of later cell division. The same possibility may account for the so-called delayed mutation effect noted in other bacterial work. The eight true

alternate types may be accounted for as mutants affecting a single chain of chemical syntheses that results in the formation of a common end product.

Four mutant lines showed alternative requirements for three substances. These four lines arose like those above for the six lines having alternatives due to mixture of distinct bacterial types. Three mutant lines were of a single type requiring two metabolites. These lines were formed by irradiating mutants a second time and then separating out the second mutants to give lines having the double requirements. Two mutant lines had three metabolite requirements made again by successive irradiations and separations of the three mutant forms. One mutant line had four requirements. Four mutant lines required more than four known metabolites. The metabolites considered were adenine, arginine, cystathionine, cysteine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and antigens. The different mutants distributed for virulence and immunizing ability are shown on the scatter diagram together with the metabolite they now require.

The mutants are all located in the left hand diagonal half of figure 1. There is a high positive curvilinear correlation between the virulence of the mutant in its capacity to initiate the disease and the active immunity generated in the host due to its reaction with the mutant. Examination of the data shows that if the capacity of a mutant to cause immunity in the host is considered as increasing as the logarithm of its capacity to cause death and morbidity then a simple linear relation will exist. This relation is described by the equation

$$\textit{acquired resistance} = a + b\,(\log_{10} \textit{virulence on first contact})$$

The acquired resistance is measured as the percentage of mice which survive the challenge dose of 11C *S. typhimurium* mutant, the a may be looked on as the average resistance to avirulent mutants at first contact with the 200,000 dose, b is the regression coefficient. The virulence on first contact is measured by the percentage dead after inoculation (plus 1 to avoid zero values) with a dose of 200,000 mutant *S. typhimurium*. The observed equation is

$$\text{acquired resistance} = 34.6 + 33.8\,[\log_{10}\%\ (\text{dead} + 1)]$$

The graph portraying the trend of acquired resistance with successive degrees of resistance is shown as the curved line on the chart. The correlation of natural and acquired resistance is 0.69 when the data are considered in this manner. Measured on the scale of variance controlled, there is a gain in estimating acquired immunity of 37 percent when the estimate is made from evidence on natural virulence of the organism.

Comparison of the average trend line with the original observations shows that most of the trend variation in the data is removed by this line.

It is known that *S. typhimurium* organizes an endotoxin. The results of figure 1 could be interpreted as indicating that the quantity of endotoxin produced by the bacterial line was governed by genes in many loci. The quantity of endotoxin could create the conditions necessary for growth in the host. Mutation could affect the endotoxin production in many ways. The virulence

tests would indicate how the endotoxin was increased or decreased by mutation. The acquired resistance tests would indicate the capacity of the quantity of endotoxin generated by the bacteria to induce the immunity in the host. Endotoxin in small amounts would raise the acquired resistance a small amount. Increasingly greater amounts would increase the acquired resistance by the logarithm of the amount rather than as a directly additive increment. Endotoxin dosage could be considered as measured by the virulence tests. Immunizing effects can be titrated by the acquired resistance observed after the tests. The *in vivo* virulence-immunizing reactions would then fall in the pattern of a number of toxicological substances in which the endotoxin was the agent subject to genetic modification as described by the equations presented above.

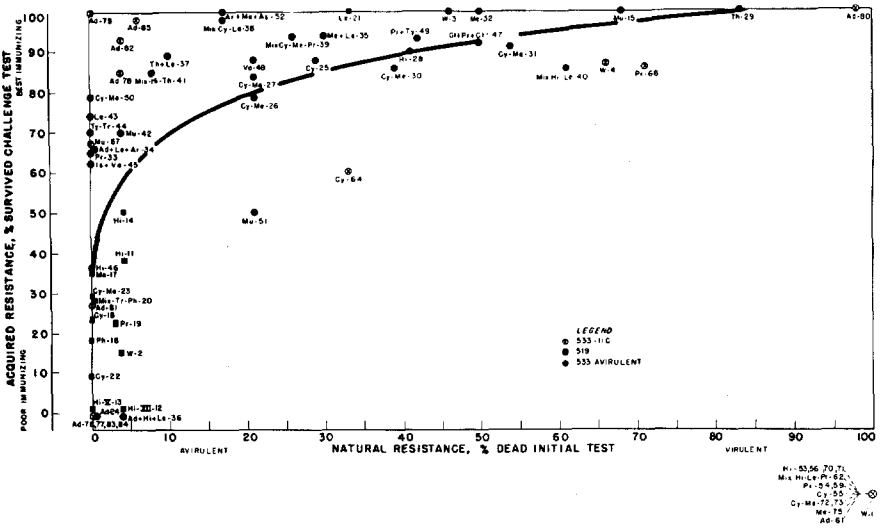

FIGURE 1.—Distribution of bacterial mutants tested for virulence and for immunizing capacity.

But the explanation is seemingly not that simple. HILL, HATSWELL and TOPLEY (1940) compared the genetic resistance to an extracted endotoxin of *S. typhimurium* as selected out of a group of mice over a number of generations with resistance to a challenge dose of intact organism. They found no correlation between the two. This result would seem to indicate that the disease syndrome has, as independent variables in the severity of the disease, capacity of the organism to grow in the host and the endotoxin produced by the pathogen. In the face of this evidence, all of the observed relations of the data in figure 1 cannot be attributed to the endotoxin effect.

Considered individually there are mutants which hold the virulence of their original parent lines and those which do not. In the right hand lower corner of the chart are 12 mutants from 533-11C. These mutants retained the virulence of the 11C (indicated by W_1) parent of 533-11C to the extent that these

mutants killed all of the mice in which they were introduced. The mutants included four which require histidine, two require proline, two cysteine or methionine, one each requires cysteine, methionine, adenine or a mixture of histidine, leucine and proline. Similarly adenine-80 and proline-68 were both more virulent than their parent 533-11C (indicated by W-4).

Several mutants lost virulence. Adenines-76, 77, 79, 81, 83, 84 and multiple-67 lost all virulence. Adenines-78, 82 and 85 lost nearly all virulence. Cysteine-64 lost half of the parent virulence following mutation. 533-11C itself has lost virulence when compared with its 11C parent, 67 percent deaths as compared to 100 percent.

Parent 533, W-3, an earlier direct transfer of 11C, lost more virulence than 533-11C. Its average virulence on test was 45 percent deaths. The drop in virulence after transfer from 11C is disturbing. In the more than 25 years that 11C culture series has been kept in this laboratory no change in virulence has been noted in the course of many tests yearly. 11C is known to mutate (ZELLE 1942) to forms showing decreased or increased virulence when separated into pure lines, but as it is kept by us has not itself reflected any changes by differences in virulence or general morphological or cultural characteristics between the successive transfers. The 533 transfers were shipped from Ames, Iowa to Amherst, Massachusetts. They were kept as stab cultures on brain heart infusion agar.

The differences in treatment are the only known selective agents which might favor particular spontaneous mutants of a greater growth capacity on culture media and possibly lower virulence. Experimental check is required to determine if this tendency to lose virulence may be accounted for by these cultural conditions.

533 was one source of mutants having different metabolite requirements. Threonine-29 was quite virulent. Threonine and leucine requiring mutant-37 had little virulence. Adenine-24 from the same source had little virulence. Other metabolite requiring mutants had virulence tests of all intermediate types.

Parent culture 519, W-2, although isolated from a human case had little virulence for our highly susceptible Ba mice. In general mutants from this parent type were likewise quite avirulent. One culture, leucine-21, alone of all the rest, increased markedly in virulence. This culture showed an even more pronounced change away from its parent's characteristics. It had the capacity to stimulate acquired immunity reactions in the host; animals which survived the initial infection with this mutant were 100 percent resistant on retest whereas only 15 percent of those exposed to the parent 519 were able to survive the challenge.

The immunizing abilities of the different metabolite mutants show the same types of variation that are observed in the initial tests for virulence. Mutants for adenine requiring 76, 77, 83, 89 of 533-11C are avirulent and poor immunizing. Mutant 81 requiring adenine and avirulent stimulates the host immune system to some degree. Adenine requiring mutants 78, 79, 82 and 85

are avirulent but have the best immunizing ability. Mutant adenine-80 requiring is virulent and immunizes solidly. Mutant-61 is so virulent that no mice survive for test of its immunizing ability. Comparisons for the other mutants and their parents bring out similar variations within other metabolite requirements.

Relation of mutant virulence to that of parent line

The data show that mutation to a given requirement may be accompanied by all degrees of change in virulence. The average change when the parent line is virulent is some reduction in virulence and a lowering of immunizing ability. The data in figure 2 bring out these facts. In figure 2 the mutants of 519 are shown as solid squares, ■, those of 533 as solid circles, ●, and those of

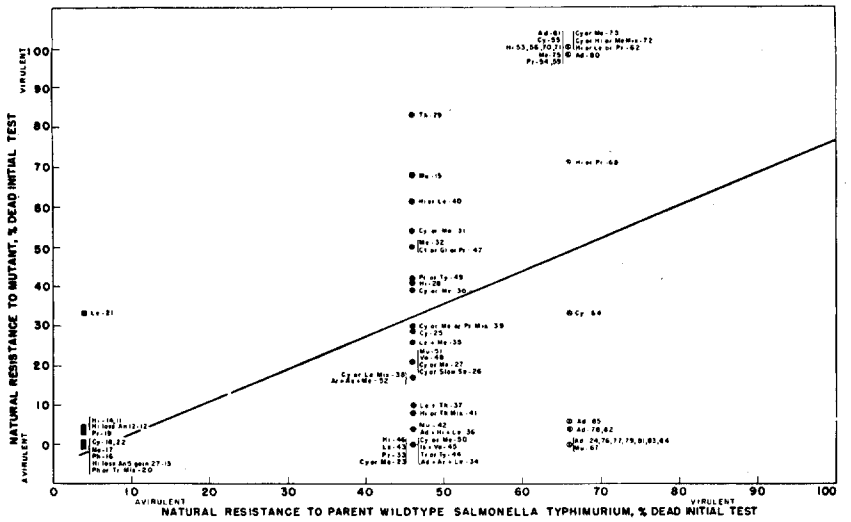

FIGURE 2.—Relation between the virulence of the parent line of *S. typhimurium* and the virulence of the mutants.

533-11C as the open circle with the cross, ⊗. The mutant's virulence plotted against that of the parent line shows that a correlation exists between the virulence of the parent and that of the mutants which occur in this line. The product moment correlation for these data is 0.45. The average trend is apparently satisfactorily represented by the linear regression equation drawn on the chart.

$$\text{Percent dead of mutant} = 0.82\,(\%\ \text{dead of parent line}) - 5.9$$

The surface contour of the parent-mutant virulence is peculiar in that the sections take J- or U-shaped forms. For 519 the mutant virulences show a clear-cut J-shape frequency distribution. Most mutants are without virulence. Only one, leucine-21, has a greater virulence than the parent. 533 mutants show the same type of effect but the J is less extreme. Most mutants are toward no virulence. Only seven mutants have greater virulence than the

parent. The mutants of 533-11C show a U-shaped frequency distribution. They are either completely virulent or are avirulent. Only two mutants out of the total number, 26, display sure intermediate virulence. The parent type had the highest virulence of those under test. Several types of nutritional mutants are noted in the virulent group. The avirulent mutants were largely adenine mutants, but this may have no particular meaning since there are also adenine mutants at the high virulence end of the distribution. The character of the surface contours of figure 2 makes it clear that no particular emphasis is to be put on the exact value of the correlation.

The data indicate that in general the observed effect of mutation on virulence is directional in that, even though the most susceptible mouse strain known to us was the test animal, the avirulent strains, if they are to survive,

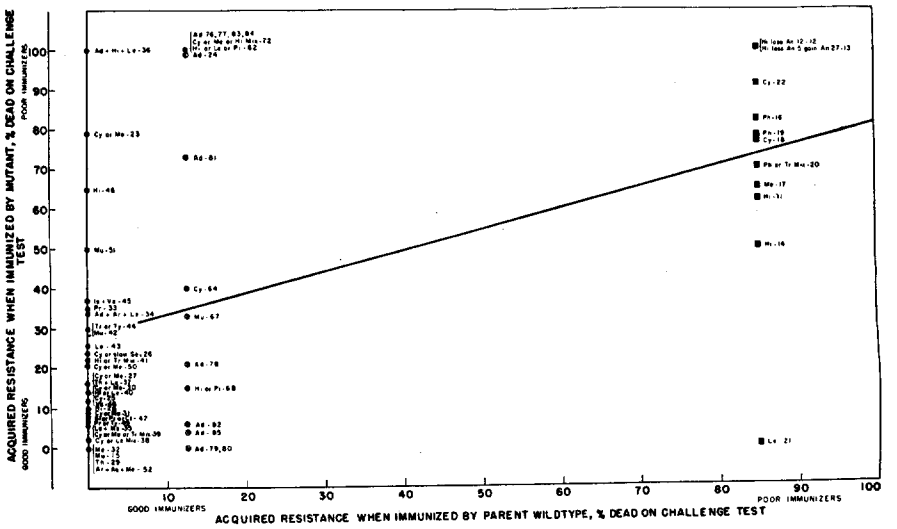

FIGURE 3.—Resistance acquired by mice exposed to the various mutants plotted against that of their parent lines as measured by percent dead on challenge by *S. typhimurium* 11C.

in a sense have only one direction in which the virulences may change, they must increase. Similarly the virulent parents have more opportunity to decrease than to increase in virulence. Increases occur but in most instances the direction of change is toward lesser virulence. These facts conform to those brought out for *Phytomonas stewartii* some years ago by LINCOLN and GOWEN (1942) in their study of the same problem.

Relation between mutant immunizing power and that of its parent line when challenged by virulent 11C

The immunizing powers of the different mutants are plotted against those of their parent lines in figure 3. The significance of the symbols is the same as with the previous charts. As an immunizer 533 proved better than 533-11C. Both were distinctly better than 519. Comparison of figures 2 and 3 shows

that 533 and 533-11C have changed their order. In virulence 533-11C had greater virulence than 533 whereas for immunizing ability 533 stimulated greater immunity than 533-11C. The differences between these lines are not great in either case but they do suggest that lethality and immunizing power are not completely correlated. This fact is further substantiated by a comparison of the individual mutants.

The mutants of 533 range in immunizing power from good to poor or none. The distribution of the mutants is again J-shaped. The larger number of mutants fall in the good immunizer class as did the parent line. A few of the mutants, notably the multiple adenine plus histidine plus leucine-36 and the alternate cysteine or methionine-23, have had their particular metabolite mutations accompanied by sudden losses in immunizing power. These mutants show that the particular changes may effect immunizing power in several ways.

533-11C mutants show similar variations although in general the adverse effect of metabolite mutation on its subsequent immunizing ability is more extreme.

The mutants of the poor immunizer line 519 show a range in immunizing ability. In general immunizing ability increases when mutation occurs in this strain—one type, leucine-21, is the best and most striking example of this change.

There is a correlation between the immunizing ability of the parent line and that of the mutants. The average trend for the mutants is given by the straight line. The correlation value 0.49 and the equation of the line

$$\%\ \text{dead on challenge of mutant} = 0.53\,(\%\ \text{dead on challenge of parent line}) - 28.2$$

are subject to the same comments as those discussed for figure 2.

Metabolite mutation generally does not affect the immunizing power of the bacteria greatly, but on occasion the change can be accompanied by a change from a good immunizer to a poor or vice versa.

Average virulence and immunizing capacity of the different metabolite mutants

The average virulence and immunizing capacity of the different metabolite mutants is shown in the following scatter diagram, figure 4.

The distribution of the metabolite requiring mutants again brings out the correlation between virulence and active immunizing ability.

A general characteristic of the mutants is a regression from the parent type of virulence or of immunizing ability.

Figure 4 shows that the average virulence of the different metabolite mutants is less than that of the parent types, W-1 and W-4. Only cystathionine and proline mutants had greater virulence than parent type 3, the other 14 metabolite mutants displayed less virulence. The *S. typhimurium* parent isolated from a human case was exceeded in virulence and in immunizing capacity by most of the mutants.

The cystathionine, cysteine, histidine, methionine and proline mutants retained medium virulence. The phenylalanine, tryptophan, isoleucine, arginine and valine mutants were nearly avirulent. These mutants differed in their immunizing powers, however. The arginine, valine and isoleucine mutants had nearly as good immunizing power as the more virulent cystathionine, methionine, cysteine or proline mutants, whereas the phenylalanine mutants were poor immunizers for the 11C challenge organism.

Mutants with different chemical requirements have average differences in their pathogenic reactions but as with the individual mutants a certain amount of randomness in the effect of the mutation on virulence and immunizing capacity appears evident.

Table 8 presents an analysis of the virulence reactions.

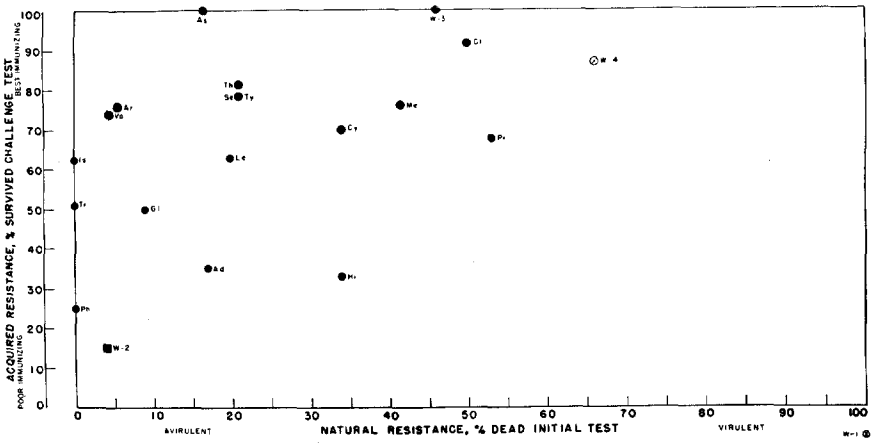

FIGURE 4.—Average virulence and immunizing capacity of the different *S. typhimurium* mutants and parental types.

There were 17 different metabolite requirements for the mutants in these data. Some mutants required two or more of the metabolites for growth. For the analysis, these mutants were considered as separate requirements. The approximations are minor with little effect on the biological interpretations to be drawn from the data.

In this method of analysis the interaction and joint effects of two or more mutations affecting different requirements are not adequately separated. These joint effects must be small. Analysis of that portion of the data in which each mutant has but one known change in its metabolite requirements leads to mean squares which are directly comparable with those of the whole data. The biological conclusions to be drawn from the sets of data are the same. The distinction between the bacterial mutant lines which have but one mutation as against those which have more may be more apparent than real. Only a limited number of metabolites out of the possible number required by the mutants of *S. typhimurium* are or can be tried out. The number of mutants observed is a function of the techniques for separation as well as of the rate of mutation. As a

TABLE 8

Variance of bacterial mutants in virulence—First test. Ba mice. Metabolites.

Source of variance	d.f.	Sum of squares	M.S.
	Mutants with single known requirement		
Among metabolite requirements	8	31	3.90
Within metabolite among lines	30	204	6.81**
Within metabolites and lines	1361	47	0.03
	All mutants		
Among metabolite requirements	16	51	3.21
Within metabolite among lines	77	392	5.08**
Within metabolites and lines	3084	188	0.06

consequence, lines where a single mutation is observed may also have other undetected mutations. The designation one, two, or more mutations is thus arbitrary, making a more complete analysis unwarranted.

Table 8 shows that the within metabolites and lines variation is small. The among metabolite and the within line requiring a metabolite variances are both large beside this standard. The metabolite mutations have changed the virulence characteristics both as to separate mutations for a given metabolite and as to mutations to different metabolites.

Comparing the between metabolites mean square with the within lines requiring a metabolite mean square shows more variation in virulence of different mutations to the same chemical requirement between mutants to differ-

TABLE 9

Variance of bacterial mutants in virulence—Challenge test. Ba mice.

Source of variance	d.f.	Sum of squares	M.S.
	Mutants with single known requirement		
Among metabolite requirements	8	18	2.20
Within metabolite among lines	22	115	5.25*
Within metabolites and lines	860	78	0.09*
	All mutants		
Among metabolite requirements	16	83	5.21
Within metabolite among lines	60	251	4.19**
Within metabolites and lines	2071	198	0.10

ent chemical requirements. This indicates that the particular chemical changes observed in the mutants may not be the primary cause in the variations in virulence observed. Rather it appears that more or less random changes in the organism occurring concurrently with mutation are the primary causes of the changes in virulence. These changes could be mutations of other genes arising simultaneously with the mutation of that for the particular metabolite, or alternatively the genes controlling a given metabolite requirement are found at several loci and these genes have multiple and different effects, the mutation of any one of which would give the metabolite requirement but a virulence different from that of another gene.

The analysis for the acquired immunity effects is presented in table 9.

The property of stimulating the host to generate active immunity shows a variation similar to that of virulence. Both the among metabolite and the within metabolite variations are highly significant. These variations are about equal. The immunizing abilities of the mutants requiring different chemicals for growth differ and differ from those of their parent strains. This suggests that immunizing ability may be affected by the particular metabolite requirement.

The fact that the different mutants for the same chemical requirement show variations like those between the different chemical requirements makes it evident that the physiological change in the chemical requirements is not the only cause of the alteration in the property of inducing active immunization. Genetically the mechanism required to explain the observed result would appear to be some combination of the following hypotheses: that the agent, X-rays or ultraviolet, causes mutations in genes governing the formation of the substances necessary for immunization simultaneously with those governing the synthesis of the metabolites under consideration or that there be several genes for the metabolite under study and these genes have as a second attribute different effects on the synthesis of the immunizing substances. At present it has not been possible to separate these hypotheses.

DISCUSSION

The variations observed in the virulence or immunizing abilities of the different mutant strains of *S. typhimurium* tested showed a correlation between the genotypes of the lines involved and their capacities to cause moderate or extensive mortality. The mutants were distinguished by their inability to reproduce without the addition to their diets of certain metabolites not required by their parent wild types. These changes in growth requirements would be expected to affect the adaptabilities of the organisms to reproduce within a host whose body fluids may be a complete medium. The different requirements of the pathogens compete with those of the host cells in new ways. Similarly the synthesis of any endotoxic substances by the pathogens, normally generated to further penetration into the host tissues, could be altered. New substances or substances in increased amounts could be formed by the mutants. The release of these materials into the host system could lead to

changes in the pathogenic pattern. A gene responsible for alterations in the normal metabolic sequence appears to cut the successive reactions at some particular step. The end products of the incomplete synthesis could pile up. Some of these products are known to be toxic. Their presence in the host system could contribute to the severity of the disease. All of these effects and others not mentioned could be expected as a consequence of a mutation in the pathogen.

Among the results there could be some due to the direct effects of the mutations on the various component parts of the syndrome which give *S. typhimurium* its ability to cause typhoid in mice: This disease property is rather highly specific. It would appear to represent some chance combination of genes which was preserved by and improved upon in successive generations of reproduction by selection of the better gene combinations and the inclusion of any of the more favorable mutations which might have occurred. Witnessing an epidemic carries the conviction that the hereditary combination of genes in the pathogen involved is efficient. It seems unlikely that random changes of the nature of mutations could be made in the gene complex involved and often improve the disease-producing machine of the highly virulent organisms. Our results agree with this expectation. The mortalities and morbidities of the mutants of a highly virulent strain are generally less than the parent. These reductions in virulence are probably explicit for the given mutant, but are not necessarily due to the criterion on which the mutant was separated from the rest of the group. The twelve adenine mutants from 533-11C tested on fifty or more mice each are illustrative of what was observed for other mutants. Seven of the adenine mutants caused no deaths, two had 4%, one had 6%, one had 98%, and one had no survivors. Each of these mutants was separated on the basis of its effect on adenine. The range in virulence effects shows that the adenine requirement is not itself the primary cause of virulence. Rather it is some change accompanying the shift in the adenine requirement.

Where the parent culture is avirulent, any observed changes in virulence will of necessity be toward higher mortality. Our parent type 519 was nearly avirulent. Of the eleven mutants only one, that for leucine-21, showed a moderate increase in virulence. The mutations as they are observed are directional in that the mutants tend to be less virulent when their parent is virulent and more virulent when the parent is avirulent. The particular change in observed virulence is not necessarily related directly to the metabolic change by which the mutated gene was detected.

BACON, BURROWS and YATES (1950) have made a study similar to ours on the virulence of biochemical mutants in *Bacterium typhosum* when introduced into commercial mice. The mice were treated in groups. One received 50 million organisms. The other mice were divided into groups injected with a range of doses to cover the ALD dose estimate for probit analysis. Their results agree in many respects with our own data. The majority of their mutants did not change in virulence. In their minor test on 10 mice the different mutants to the same requirement varied considerably in their virulence. This is in line

with the conclusion drawn above that the particular character on which the mutant was separated is generally not the primary cause of the virulence change. Rather it is the place in the chain of syntheses that the gene affects the organism or the accompanying mutations which leads to the observed changes in virulence. Aspartic acid requiring mutants and purine or purine + aneurin mutants are consistently mutants of lowest virulence in the BACON et al. (1950) experiments. In our data there is but one aspartic acid requiring mutant and that mutant also requires arginine and methionine. This mutant showed a decrease in virulence of 66 percent. The adenine mutants in our data retain the parent virulence or show decreases in virulence depending on the mutant. As within our own data so with the comparison of our data with those of BACON, BURROWS and YATES (1950), the mutant characters used in identifying the mutant are correlated with rather than causative of the changes in virulence observed.

Natural mutations toward lower or higher virulence can and do occur as demonstrated in the work of ZELLE (1942) on *S. typhimurium* in our mice; GOWEN, STADLER and BELL (1937–1947) on *S. gallinarum* in fowl; and WELLHAUSEN (1937) and LINCOLN and GOWEN (1942) in *Phytomonas stewartii* in maize. Mutation in virulence following X-radiation have been quantitatively estimated in *Phytomonas stewartii* by LINCOLN and GOWEN (1942). These results are concordant with those in this study in that they show that the capacity to mutate toward or away from increased virulence is observed. The frequencies of the changes depend on the parent type virulence and the stability of the organism. Immunization experiments by GOWEN (1945) have shown that the capacity to stimulate active immunity is likewise affected by the mutations.

In certain of these mutations colony morphology or other characteristics have been seen to vary with the mutations in virulence. These changes are again correlative changes rather than causative over the whole range of effects.

SUMMARY

Sixty-seven metabolite mutants of spontaneous, X-ray or ultraviolet induced origin are tested and analyzed for their virulence and capacity to stimulate active immunity in mice.

The mutants were from three parent stocks; 519 derived from a human case of *S. typhimurium* infection and of low virulence to mice, two derivatives of our strain 11C pathogenic to mice in proportion to the particular host genotypes in which these organisms are introduced.

Four inbred and selected strains of mice were used in these experiments. These mice behaved consistently both within the strains and among the experiments and sexes.

The data presented are largely for the most susceptible mouse strain as the data from them are most informative. The mortalities or active immunizing abilities as tested in the different strains are correlated but the mean survivals change with the strains.

The different mutant bacterial lines showed significant differences in their virulence and in their immunizing capacity. These lines have greatly extended the known mutative changes in pathogenicity and are furnishing significant material for the further analyses of this problem.

A curvilinear correlation, accounting for some 37 percent of the observed variation in active immunizing power when the virulence is known, is found between the virulence of the mutant and its power to stimulate active immunity.

The virulence of the mutant is related to the virulence of its parent. A parent of high virulence tends to have mutants of lesser virulence, a parent which is avirulent tends to have mutants of greater virulence than the parent.

Similar reactions are noted for the capacity of the mutants to stimulate active immunity. Parents having poor immunizing powers tend to have mutants which are better, but not much better, immunizers. Similarly the parent having good immunizing powers tends to have mutants with somewhat less good active immunizing stimulating capacity.

The virulence and immunizing capacity of the different nutrient requiring mutants differ more than would be expected on random sampling. However, the variation within the metabolic mutants of like requirement is greater than either that among metabolites or within metabolites and lines. These differences are interpreted as indicating that the particular chemical changes observed in the mutants may not be the primary causes of the variations observed in either virulence or immunizing capacity. The observed variations could be due to mutations of other genes arising simultaneously with the particular metabolite mutant or that the genes controlling a given metabolite requirement are found at several loci and these genes have multiple or different effects—the variation of any one of which would give the metabolite requirement but a virulence or immunity different from that of another gene.

The mutation process, which led to a line having a phenotypically different metabolic requirement from that of its parent as studied herein, had no consistent influence on either virulence or active immunizing ability of the new bacterial line.

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