THE EFFECTS OF BIOCHEMICAL MUTATION ON THE VIRULENCE OF *BACTERIUM TYPHOSUM*: THE VIRULENCE OF MUTANTS.

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A PREVIOUS paper (Bacon, Burrows and Yates, 1950) described the induction, selection and characterization of a number of biochemical mutants of *Bact. typhosum* derived primarily for the purpose of studying the effects of biochemical mutation on virulence. Some general aspects of the complex problem of virulence in bacteria have been included in a recent review by Watson and Brandley (1949). The absence of a convenient test animal readily susceptible to infection by small numbers of typhoid organisms has been a considerable handicap to the progress of experimentation on typhoid. Nevertheless the method developed by Felix (1938), using organisms suspended in Ringer's solution and injected intraperitoneally into mice, has proved useful in differentiating strains of typhoid with respect to virulence, despite the fact that relatively large numbers of the most virulent organisms are required to induce a fatal intection in this animal.

It is, however, possible with the aid of hog gastric mucin to reduce the numbers of typhoid organisms capable of initiating the fatal infection of mice to a very low figure (Review: Olitski, 1948). Batson, Landy and Brown (1950) in a recent comparison of the efficiencies of methods employing intraperitoneal injection into mice of organisms suspended in saline or in mucin, or the injection of saline suspensions intracerebrally, concluded that, of the three methods, greatest differentiation of typhoid strains with respect to virulence was obtained by that employing mucin.

Although of value in differentiating strains of lower virulence from one of high virulence, the very small numbers of organisms comprising an average lethal dose of the latter suspended in mucin precludes the use of this adjuvant for the detection of strains showing increased virulence over that of a highly virulent strain taken as a standard. The parent strain Ty22 from which our mutants were derived is of a high virulence, and as it was considered possible that some mutants may show enhanced virulence, the use of mucin was avoided in this work.

Most workers are in agreement that strains of *Bact. typhosum* showing the highest virulence for mice are culturally smooth, relatively opaque and possessing maximum amounts of both Vi and O somatic antigens, whilst rough strains or those devoid of either Vi or O show considerably lowered virulence. To determine whether biochemical mutation materially affected the production of these two antigens considered essential for maximum virulence, the agglutination of our mutants by typhoid Vi and O antisera was examined concurrently with tests of virulence.

MATERIALS AND METHODS.

Media.

Tryptic digest meat broth (TMB) or agar (TMA) were employed as complete medium. Glucose-ammonium-phosphate medium prepared as previously described (Bacon *et al.*, 1950) was used as minimal medium. Phosphate buffer contained 4·5 g. KH_2PO_4 per litre and was adjusted to pH 7·4 with NaOH.

Strains.

The strains used were biochemical mutants derived from a single ammonia assimilating strain Ty22 subjected to X-rays, ultra-violet irradiation or to nitrogen mustard. In all some 93 such mutants representing 25 different types have been examined. They are listed together with their growth factor requirements in Table II. Cultures were maintained in the dry state by the gelatin-ascorbic acid method described by Stamp (1947) and stored *in vacuo* at $\pm$ 2° C.

Suspensions.

A standard procedure was adopted for the preparation of all suspensions used for virulence experiments. Dried pellets were emulsified in TMB, plated on TMA to give an area of confluent growth and a number of separate colonies, and incubated 16 hr. at 37° C. The plates were examined for purity, refrigerated 7 hr., a representative inoculum from the edge of the confluent growth seeded to minimal agar supplemented with optimal amounts of the necessary growth factor(s), and incubated 18 hr. at 37° C. in *ca.* 5 per cent CO_2 95 per cent air. After cooling in the refrigerator for 30 min. the growths were emulsified in ice cold buffer to the opacity required for injection, viable counts made on these suspensions by the method of Miles, Misra and Irwin (1938), and the suspensions injected into mice with the minimum of delay.

Agglutinating suspensions were similarly prepared, using normal saline at room temperature in place of cold buffer.

Virulence tests.

White mice (Hough strain) equally divided as to sex and between weight limits of 18 to 22 g. were used throughout. They were received in batches of 200 to 400 and assigned to separate containers the day before injection. The required dose contained in 0·5 ml. was injected intraperitoneally and the animals observed over a seven-day period. Two scales of testing were used :

(a) *Minor tests.*—All mutants were subjected to a minor virulence test in which 50 million cells of each mutant, estimated on an opacity basis, were injected into each of 10 mice. Six to 18 mutants, buffer and Ty22 controls were assayed per experiment, and those mutants apparently altered in virulence on the evidence of such tests were selected for the major tests. Limitation in the availability of test animals made larger scale testing of all mutants impracticable within a reasonable period. Major tests were therefore limited to those mutants appearing altered in virulence in the minor tests and to one representative of each different type.

(b) *Major tests.*—Eighty mice were used for each mutant. One to three mutants were assayed against Ty22 per experiment, using four graded doses, estimated from smaller scale tests to cover the A.L.D. of each culture, each injected

into 20 mice. Probit mortality in 7 days–log dose regression lines were constructed and the relative virulence of the mutants with respect to Ty22 determined by the methods of probit analysis. Experiments in which more than one of the 20 buffer control mice died were repeated. Of 580 control mice used throughout these tests no deaths were recorded within the first 5 days of the experiments, and a total of only 4 within the seven-day periods. Deaths among control mice occurred only in experiments conducted between January and March of the 11 months' duration of these experiments.

Agglutination tests.

Living agglutinating suspensions were standardized to 2300 million organisms per ml. (opacity 5 of Burroughs Wellcome scale), and one drop added to 1 ml. of the Vi or O serum dilutions in 5 cm. × 1·2 cm. tubes. The tubes were incubated 2 hr. at 37° C. in an incubator and read after standing overnight at room temperature. Suspensions killed by heating 30 min. in a boiling water bath were similarly examined for their O-agglutinability.

RESULTS.

General characters of mutants.

The majority of mutants were colonially indistinguishable from the parent Ty22. They grew readily on TMA, producing circular, smooth, more or less opaque brownish colonies. The exceptions noted were mutants 9749 and 20085 (histidine requiring) giving less smooth colonies, and mutant 20092 (guanine or xanthine) giving normal smooth colonies about one-half the diameter of those of Ty22. Aspartic-less mutants gave colonies with a distinctive annular depression.

Similarly the majority of mutants were morphologically indistinguishable from the parent. The variations noted among those dissimilar were 9749, in which the cells were shorter and aggregated into streptococcus-like chains, 20085 with many long unsegmented filamentous forms, 20037 (methionine) a plump coccobacillus, 20058 (cystine) and 20092 both occurring as plump coccobacilli, many in short chains.

Phage sensitivity.

Without exception the mutants were fully sensitive to lysis by Type IIE typhoid Vi phage used in its critical test dilution for Ty22, when tested by the plate method described by Craigie and Yen (1937).

Virulence of mutants.

Table I is illustrative of minor tests of virulence in which mutant cultures were injected into 10 mice, each receiving a dose of 50 million organisms estimated on an opacity basis. Although all injected suspensions were standardized to the same opacity, their viable counts varied more than could be attributed to limitations in the accuracy of opacity determination. This discrepancy between the expected and apparent viable count made the interpretation of virulence tests a matter of some difficulty. The question that arose was whether similar doses of different organisms were better represented by equal volumes of suspension of equal opacity or by equal numbers of viable organisms. Presumably it is the mass of living bacterial matter injected rather than the numbers of individual

or groups of organisms into which it is divided that is of primary importance in a suspension used in a test of virulence. Measurements of opacity would take into account variation in the morphology of the organisms in suspension without differentiating between living and dead or between organisms of differing transparency, whereas viable counts make no allowance for differences of morphology, and can only be reliable when all cells are distinct individuals. Thus neither method of estimating the dose of living bacterial matter administered is fully reliable when comparing the virulence of suspensions of different organisms which may differ morphologically or in the transparency of individual cells.

As it is improbable that the opacity measurements would be greatly in error from the presence of dead cells in physiologically young cultures, whilst viable counts are considerably affected by aggregation or variation in the size of cells, we consider the relative virulence assessed on opacity dosages is the more reliable. Assessments were made on both bases, but only those on an opacity basis are recorded in the tables.

In the majority of tests Ty22 injected at a level of 50 million organisms (opacity) resulted in 8 out of 10 fatal infections. This number varied on occasions between 4 and 10 out of 10. The variation noted may have been the result of normal sampling fluctuations, of change of virulence of Ty22 or of change in resistance to infection of the mice. On occasions when Ty22 killed a low or high proportion of the mice, those mutants which we have reason to believe are not significantly altered in virulence tested in the same experiments also killed a low or high proportion, suggesting a general higher or lower resistance of the animals to infection rather than a change in virulence of the standard Ty22. The two experiments recorded in Table I illustrate this point.

From these two experiments mutants 20063, 20025 and 20085 were regarded as altered in virulence and subjected to major tests. Mutant 20071 was reassayed in a subsequent minor test.

TABLE I.—*Illustrating Minor Tests of Virulence.*

Expt. number.	Culture.	Growth factor.	Dose organisms × 10^{-6}. On opacity measurement.	Dose organisms × 10^{-6}. On viable count.	Number of mice injected.	Deaths in— 3 days.	Deaths in— 7 days.
31	(buffer)	..	—	—	10	0	0
	Ty22	—	50	69	,,	4	4
	20063	PAB	,,	49	,,	0	0
	20022	Phenylalanine	,,	67	,,	5	5
	20045	Threonine	,,	56	,,	6	6
	20068	Cytosine	,,	42	,,	6	6
	20025	Leucine	,,	47	,,	1	1
	20075	Glycine	,,	65	,,	6	6
37	(buffer)	..	—	—	10	0	0
	Ty22	—	50	53	,,	8	8
	20074	Aneurin	,,	53	,,	9	9
	20105	,,	,,	47	,,	8	8
	20110	Histidine	,,	54	,,	9	9
	20085	,,	,,	13	,,	0	0
	20073	,,	,,	50	,,	8	8
	20087	Methionine	,,	59	,,	8	8
	20084	Proline	,,	54	,,	9	9
	20071	Cystine or methionine	,,	60	,,	10	10

TABLE II.—*List of Mutants with their Growth Factor Requirements and Numbers of Mice Killed out of 10 by Doses of 50 Million Organisms.*

Growth factor.	Mutant.	Deaths. Mutant.	Deaths. Ty22.
Methionine	3306	7	8
	4438	8	8
	5459	8	8
	7010	7	8
	20012	5	8
	20015	9	8
	20018	7	7
	20023	8	10
	20031	8	10
	20037	9	10
	20056	9	10
	20072	9	10
	20083	5	7
	20087	8	8
	20114	9	10
Cystine	2651	8	9
	6569	8	9
	6721	8	9
	20021	8	8
	20040	4	4
	20058	0	10
	20059	6	6
	20120	6	4
	20125	8	4
Cystine or methionine	20062	9	10
	20071	6	6
Arginine	6775	9	9
	7226	5	9
	20028	6	6
	20046	8	4
	20069	9	10
Leucine	20025	1	8
	20082	1	9
	20113	4	9
Histidine	3160	5	8
	3663	3	8
	5555	4	8
	6346	7	8
	9749	0	8
	20030	8	10
	20065	4	10
	20073	8	8
	20085	0	8
	20110	9	8
Proline	9468	9	9
	11392	6	8
	13302	9	10
	16891	9	8
	20011	5	4
	20067	2	4
	20084	9	8
	21857	9	8
Glycine	7072	7	9
	10833	8	10
	20034	9	10
	20075	6	4
Glycine or serine	20066	9	10
Lysine	3234	7	9
	7330	9	9
	12190	8	10
Valine + isoleucine	7168	8	10
	9355	9	8
	12559	4	8
Threonine	20045	6	4
	21403	8	8
Aspartic acid	8505	0	8
	19341	0	8
	19662	0	8
Phenylalanine	20022	5	4
Tyrosine	20100	7	7
Valine + isoleucine + pantothenate	16265	9	8
	20026	2	6
	20078	6	6
	20096	4	4
Pantothenate	10020	9	10
Aneurin	20002	6	8
	20032	9	7
	20039	4	6
	20074	9	8
	20105	8	8
Biotin	13461	5	8
	20053	7	7
Nicotinamide	8974	9	10
	20035	6	7
PAB	20063	0	4
Cytosine	20057	8	7
	20068	6	4
	20112	7	8
Purine	20080	0	8
Guanine or xanthine	20092	0	8
Purine + aneurin	479	0	8
	20095	0	6
	20102	0	8
?	20001	4	8

Table II summarizes the results of minor virulence tests of all our mutants, giving the numbers of mice killed by the mutants in comparison with the numbers killed by an equal (opacity) dose of Ty22 tested in the same experiment.

An illustrative major virulence test is presented in Table III. Tables IV and V summarize the results of all such tests.

TABLE III.—*Illustrating Major Tests of Virulence.*

Culture.	Growth factor.	Dose organism × 10^{-6}. Opacity.	Viable.	Mice injected.	Deaths in— 3 days.	7 days.	Relative virulence with limits (P = 0·05).
Ty22	..	10	7·0	20	7	8	1·0
		20	14·0	„	11	12	
		40	28·0	„	13	13	
		80	56·0	„	17	17	
20040	Cystine	10	10·0	„	5	5	0·72
		20	20·0	„	6	6	
		40	40·0	„	14	14	(0·43–1·1)
		80	80·0	„	17	17	
20084	Proline	10	9	„	3	3	
		20	18	„	9	9	0·64
		40	36	„	12	13	(0·38–1·0)
		80	72	„	15	15	
20080	Purine	1000	600	„	1	3	0·005
		2000	1200	„	5	6	(0·003–0·009)
		4000	2400	„	11	11	
		8000	4800	„	17	17	

TABLE IV.—*Virulence of Representative Mutants of Each Growth Factor Type.*

Growth factor(s).	Mutant.	Relative virulence with limits (P = 0·05).	
Methionine	5459	0·8	0·5 –1·2
Cystine	20040	0·7	0·4 –1·1
Cystine or methionine	20071	1·3	0·8 –2·2
Arginine	6775	1·2	0·7 –1·7
Leucine	20025	0·4	0·2 –0·6
Histidine	6346	0·5	0·3 –0·8
Proline	20084	0·6	0·4 –1·0
Glycine	20034	0·4	0·3 –0·7
Glycine or serine	20066	0·6	0·4 –0·9
Lysine	3234	1·4	0·8 –2·5
Valine + isoleucine	9355	0·6	0·3 –1·2
Threonine	20045	1·2	0·7 –2·0
Aspartic acid	8505	0·1	0·06–0·2
Phenylalanine	20022	1·4	0·7 –2·7
Tyrosine	20100	1·1	0·7 –2·0
Valine + isoleucine + pantothenate	16265	0·7	0·5 –1·1
Pantothenate	10020	1·3	0·9 –2·6
Aneurin	20039	0·7	0·3 –1·3
Biotin	20053	0·5	0·3 –1·2
Nicotinamide	8974	1·2	0·6 –2·2
PAB	20063	0·1	0·08–0·2
Cytosine	20057	0·8	0·5 –1·2
Purine	20080	0·005	0·003–0·009
Guanine or xanthine	20092	0·03	0·02–0·06
Purine + aneurin	479	0·03	0·01–0·04

TABLE V.—*Virulence of Mutants Estimated from Minor Tests to have Altered in Virulence.*

Mutant.	Growth factor(s).	Relative virulence with limits (P = 0·05).	
9749	Histidine	0·05	0·03 –0·07
20085	„	0·2	0·1 –0·3
20058	Cystine	0·02	0·01 –0·03
8505	Aspartic acid	0·1	0·06 –0·2
19341	„ „	0·1	0·06 –0·2
19662	„ „	0·1	0·09 –0·2
20063	PAB	0·1	0·08 –0·2
20080	Purine	0·005	0·003–0·009
20092	Guanine or xanthine	0·03	0·02 –0·06
479	Purine + aneurin	0·03	0·01 –0·04
20095	„ „	0·01	0·004–0·02
20102	„ „	0·03	0·02 –0·05

In vivo *reversion of mutants.*

In view of the ease with which certain mutants were known to lose their growth factor requirements in *in vitro* studies it was important to consider their stability *in vivo*. In earlier experiments organisms were re-isolated on TMA from the spleens of animals surviving the injected dose of mutant cultures on the seventh day after injection, and tested for their dependence on added growth factors for development on minimal medium. Typhoid organisms were invariably recoverable from the spleens after this time. Twenty single colonies taken at random from the TMA-plates were spot inoculated to minimal medium plates with and without added growth factor(s). After two days' incubation, growth of all inocula on supplemented minimal medium but of none on unsupplemented medium was indicative of no appreciable reversion to the non-exacting condition. Isolations from 34 mice injected with different mutant cultures were all found to have retained their growth factor requirement.

It was, however, of greater importance to observe the proportion of reverted organisms in mice succumbing to infection rather than in surviving animals. Accordingly, in subsequent experiments, animals on the point of death on the first or second days following injection were killed by cervical dislocation, a loopful of peritoneal fluid emulsified in minimal medium, serially diluted and plated on growth factor supplemented and unsupplemented minimal medium. A comparison of the numbers of colonies developing on the two media allowed a good estimate to be made of the maximum ratio of reverted to mutant organisms. Isolations made in this manner from moribund mice almost invariably yielded pure cultures of typhoid organisms. Similar isolations from dead animals were frequently heavily contaminated with coliform bacteria. Of 59 isolations from moribund mice injected with 33 different mutant cultures, 4 showed *ca.* 100 per cent reversion (mutants 9749 and 20058), 1 showed 9 per cent (20092), 5 between 1 per cent and 0·01 per cent, and the remaining 49 isolations less than 0·01 per cent reversion to the non-exacting form. The low incidence of reversion observed suggests that the majority of our assessments of the relative virulence of mutants with respect to Ty22 is unlikely to be subject to any great error from *in vivo* instability of mutants.

TABLE VI.—*The Serological Behaviour of Selected Mutants of High and Low Virulence.*

	Culture.	Growth factor.	Agglutination. Living suspensions. Vi serum dilution. 100	200	400	800	1,600	3,200	6,400	Living suspensions. O serum dilution. 250	500	1,000	2,000	4,000	8,000	16,000	32,000	64,000	Heat-killed suspensions. O serum dilution. 250	500	1,000	2,000	4,000	8,000	16,000	32,000
	Ty22	—	6	6	6	6	4	1	—	2	—	—	—	—	—	—	—	—	6	5	5	4	4	3	1	—
Mutants of high virulence	20087	Methionine	6	6	6	6	5	1	—	4	4	1	1	—	—	—	—	—	6	6	5	5	3	1	—	—
	20032	Aneurin	6	6	6	6	5	2	—	5	4	3	1	—	—	—	—	—	6	5	5	4	2	1	1	—
	8974	Nicotinamide	5	5	5	5	4	1	—	—	—	—	—	—	—	—	—	—	6	6	5	3	2	—	—	—
	20057	Cytosine	6	5	5	3	3	3	1	5	5	4	2	1	—	—	—	—	6	6	5	4	2	1	1	—
	20084	Proline	6	6	6	5	5	2	—	5	5	2	1	—	—	—	—	—	6	6	6	4	2	1	—	—
	20100	Tyrosine	6	4	4	3	1	—	—	5	3	2	1	—	—	—	—	—	6	6	6	5	2	1	—	—
	10020	Pantothenate	5	5	5	5	3	1	—	1	—	—	—	—	—	—	—	—	6	6	5	3	2	—	—	—
Mutants of low virulence	20063	PAB	5	5	4	3	2	1	—	1	1	1	—	—	—	—	—	—	6	6	4	3	2	1	1	—
	8505	Aspartic	6	6	6	6	5	2	—	3	1	—	—	—	—	—	—	—	6	5	5	4	4	3	3	—
	19321	„	5	5	5	5	3	2	—	4	4	2	2	—	—	—	—	—	6	6	4	3	2	—	—	—
	20080	Purine	6	6	5	3	3	1	—	5	1	—	—	—	—	—	—	—	6	5	5	3	2	1	—	—
	20092	Guanine	6	5	5	3	1	—	—	2	1	—	—	—	—	—	—	—	6	6	5	4	2	1	—	—
	479	Purine + aneurin	6	6	6	6	5	2	—	3	2	1	—	—	—	—	—	—	6	5	5	4	3	3	3	—
	20102	„ „	6	4	4	3	1	—	—	5	5	2	1	—	—	—	—	—	6	6	6	5	3	1	—	—
Standard: Vi	T6S	—	6	6	5	5	2	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
O	0901	—	2	1	—	—	—	—	—	6	6	6	5	3	2	1	—	—	6	6	6	4	2	1	—	—

Degrees of agglutination: 6 = Agglutination complete, supernatant clear; 5 = agglutination almost complete, supernatant turbid; 4 = agglutination just visible with naked eye; 3 = agglutination easily visible with lens; 2 = trace visible with lens; 1 = faintest trace visible with lens.

Agglutination of mutants.

With the exception of those strains referred to below, all mutants, on the evidence of the agglutinability of living suspensions by a Vi antiserum and of heat-killed suspensions by an O antiserum, were found to be of the Vi + O type. Living suspensions resisted O-agglutination in varying degrees. The O-resistance of naturally occurring Vi + O strains is commonly regarded as an index of their virulence for mice, the less O-agglutinable strains being the more virulent. However, with our artificially selected biochemical mutants the behaviour of living suspensions towards our O-antiserum was not always indicative of their relative virulence. A selection of a number of virulent and relatively avirulent mutants with their serological behaviour is presented in Table VI to illustrate the difficulty of differentiating the two types from serological evidence alone.

Three strains differed in their agglutination responses from the majority. Strains 9749 and 20085 produced slightly unstable suspensions in 0·85 per cent saline from growths on supplemented minimal medium. Grown on TMA and suspended in 0·1 per cent saline both were sufficiently stable to indicate strain 9749 to be of the Vi + O type, but less rich in these antigens than Ty22, and 20085 to be markedly deficient in O antigen. Mutant 20058 agglutinated in our Vi antiserum to a distinctly lower titre than all other mutants. These three mutants are altered morphologically and of lowered virulence.

DISCUSSION.

At the outset of this work it was visualized that biochemical mutation may possibly result in an enhancement of the virulence of our organism. The accumulation of synthetic products whose further metabolism is prevented by mutational blockage is well known in studies of biochemical mutants in fungi and bacteria. Such intermediate metabolites may well be structurally dissimilar from those of the host, resulting in the blockage of important synthetic mechanisms in a competitive manner and facilitating the establishment of a fatal infection by the mutant. Secondly, it is conceivable that an organism with a particular growth factor requirement would, on injection into a host, compete with it for that factor. Further, by nature of its larger surface area/volume ratio the organism could conceivably absorb this factor from the body fluids more efficiently than the host cells, resulting in the starvation of the latter with respect to the factor. If this were normally in limited supply and vital for the correct functioning of the cells, such starvation would probably lead to a lowering of resistance to infection and be reflected in an apparent increase in virulence of the parasite, providing that the mutation had not simultaneously decreased the resistance of the organism to the normal anti-bacterial mechanisms of the host.

Our results concerning the virulence of biochemical mutants representing a number of different growth factor types do not lend support to these views as applied to the infection of the mouse with *Bact. typhosum* mutants. However, it is possibly the case that growth factor dependence may well enhance the virulence of an organism initiating a less acute disease, by creating zones of growth factor deficiency in the microenvironment of organisms lodged in the host, rather than of one initiating a rapidly fatal toxaemia, such as follows the injection of large numbers of typhoid organisms into the mouse.

Probit analysis revealed no significant divergence from parallelism or any

evidence of heterogeneity between the regression lines of parent and mutants throughout the entire range of virulence encountered, suggesting the mutants to exert a similar pharmacological effect to that of Ty 22.

Surveying the results of major virulence tests with the supporting evidence of minor tests, it is evident that the majority of our mutants have not significantly changed in virulence. Of amino-acid requiring mutants slight lowering of virulence is associated with dependence on added leucine, histidine or glycine. An aspartic acid requirement, however, is seen to result in 3 out of 3 instances in a marked decrease in virulence. Two out of 10 histidine and 1 of 11 cystineless mutants (9749, 20085 and 20058) differ from the other mutants with these requirements in being of considerably lower virulence than the parent. Loss of virulence here may be a direct result of biochemical mutation affecting a particular step in the synthesis of histidine or cystine, or alternatively may arise from a simultaneous " non-biochemical " mutation, leading to the antigenic, colonial or morphological variations previously described.

Among 15 mutants with requirements for vitamins of the B group, loss of virulence is confined to the one mutant unable to synthesize para-amino-benzoic acid. Here loss of virulence is not associated with any morphological change or demonstrated major antigenic differences from the parent.

The most notable loss of virulence is associated with those mutants deficient in their ability to synthesize purines. Five independently isolated mutants of this type have a relative virulence of the order of 0·005 to 0·03 of that of the non-exacting strain, so that an average lethal dose is in the region of 2000 million organisms. The relatively insignificant effect on virulence occasioned by mutation to dependence on added aneurin, shown by five such independently isolated mutants, would suggest the mutation to purine dependence is the major factor resulting in loss of virulence in those mutants having a double requirement for a purine plus aneurin.

Biochemical mutation can be visualized to lead to a loss of virulence in a number of ways. A dependence on an exogeneously supplied growth factor may limit the production of non-vital constituents of the bacterial cells, which, however, are of importance from the aspect of virulence, if such constituents require the participation of the growth factor in their synthesis. It is possible that non-vital surface complexes, important for the manifestation of virulence, are produced from excess syntheses of the vital requirements of the virulent organism. Should the growth factor of a particular type of mutant be one moiety of such a complex, in its absence due to limitation in supply or to the inability of the mutant to incorporate exogeneously supplied growth factor, such a complex may not be produced or may be formed in a non-functional state. The complete absence or the limited availability of the required growth factor in the host preventing the multiplication of the injected mutant to a toxic level presents a third possible mechanism whereby biochemical mutation could lead to a loss of virulence of the mutant.

We have in Ty22 and our mutants of low virulence an opportunity to study mouse virulent and avirulent strains of *Bact. typhosum*, which in all probability differ from each other in the presence or absence of single genes concerned with the synthesis of specific growth factors. The great loss of virulence shown by purine requiring mutants is of particular interest in that these mutants conform in colonial character, antigenic structure and Vi phage sensitivity to the accepted

criteria defining virulent strains of typhoid. Studies with our avirulent mutants, with the object of determining reasons for biochemical mutation resulting in loss of virulence, are in progress.

SUMMARY.

1. The effect of biochemical mutation on the virulence of *Bact. typhosum* was investigated, using 93 mutants representing 25 different growth factor types.

2. The majority of mutants retained full virulence.

3. Slight loss of virulence was associated with a requirement for leucine, histidine or for glycine.

4. Three independently isolated aspartic acid and one para-amino-benzoic acid mutant were reduced in virulence to *ca.* 0·1 of that of the parent.

5. Five independently isolated purine-requiring mutants were reduced in virulence to *ca.* 0·02 of that of the parent.

6. The majority of both virulent and avirulent strains was unaltered in colony form, morphology or major antigenic structure.

7. Three anomalous strains of low virulence had morphological or other variation in addition to a growth factor requirement.

8. All mutants retained sensitivity to typhoid Vi phage.

9. The O-agglutinability of mutants was not infallibly indicative of their mouse virulence.

10. Possible mechanisms leading to alteration of virulence by biochemical mutation are discussed.

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