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

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THE outcome of the infection of a particular host by a particular parasite is recognized to be dependent upon a number of variable factors. Alteration of one of these in either the infecting organism or in the infected host may consequently be expected to alter the outcome of that particular host-parasite relationship. It is evident that the synthetic processes involved in the elaboration of of the bacterial body must play a primary role in determining the properties of the mature organism and its fate on introduction into a host and the interference with such processes may well result in alterations of one or more factors affecting this relationship. It was considered of interest to investigate to what extent such interference occasioned by the mutational blockage of specific synthetic enzyme systems would be reflected in an alteration of the virulence of a pathogenic organism. The typhoid organism was shown by Gladstone (1937) to be fully virulent for mice when grown on a simple synthetic medium, and was considered to be suitable for a study of this nature.

A number of agents capable of inducing mutations with respect to the synthetic abilities of bacteria are known. Those most commonly employed are X-rays, ultraviolet light and the nitrogen and sulphur mustards. The precise mode of action of these mutagenic agents is not fully understood, but their final effects are attributed to the inactivation of inheritable determinants governing the production or specificity of enzyme systems necessary for the synthesis of essential metabolites. To permit the growth of mutant forms these essential metabolites, or growth factors, must be added preformed to a medium. In current terminology forms differing from the parent in requiring added growth factors are " biochemical " mutants.

Induced biochemical mutation in *Bact. typhosum* has been investigated by few authors. Grainger (1947) was unable to select from 1000 isolates tested any mutants differing from the parent culture in colony morphology fermentation or biochemical reactions after exposure to X-rays. Following culture in the presence of acenaphthene, Grainger and Wilmer (1947) selected a rough variant unable to ferment galactose. Kristensen (1948), by serial transfer demonstrated the ability of his strains to mutate with respect to their fermentation of various sugars. Strains of typhoid bacteria were shown by Fildes and Whitaker (1948) and Mercedes (1948) to mutate spontaneously at a low rate, from tryptophan dependence to independence.

The method of isolating mutants with decreased synthetic ability initiated by Beadle and Tatum (1941) in their pioneer work on *Neurospora* is applicable to the isolation of similar mutants of bacteria. However, it is limited in that comparatively small numbers of a mixed population of mutant and predominantly non-mutant forms can be conveniently examined. Various mitigating procedures have been described by Lederberg and Tatum (1946), Farghaly, Miller and McElroy (1948), Miller, Farghaly and McElroy (1949), Meyersberg, Pomper and Cutter (1949) and Davis (1949*b*). A notable improvement simultaneously published by Davis (1948), and Lederberg and Zinder (1948) utilizes the selective bactericidal action of penicillin on growing cells whilst leaving non-growing cells without permanent effect for the selection of biochemical mutants from very large mixed populations.

The present paper describes the methods used for induction and selection of biochemical mutants of *Bact. typhosum.*

MATERIALS.

Organism.

The strain Ty2 described by Felix (1938) and employed by Henderson (1939) was used as starting material.

Maintenance of cultures.

All cultures of parent and mutants were dried (Stamp, 1947) from growth on minimal medium supplemented by growth factors as necessary, and stored at $\pm$ 2° C. *in vacuo.*

Suspensions.

Dried cultures were emulsified in tryptic meat digest broth (TMB) and plated on tryptic meat digest agar (TMA). After overnight incubation, slopes of minimal medium supplemented as necessary were inoculated from these plates. After 18 hours' incubation the slopes were emulsified in phosphate buffer pH 7·4 (KH_2PO_4 4·5 g./litre + NaOH to pH 7·4) to the desired opacity and the suspensions obtained used without further treatment. Fresh dried cultures were used for each suspension prepared on different days to avoid subculturing with the accompanying possibility of variation.

Incubation.

Cultures were incubated unshaken at 37° C. in an atmosphere of approximately 5 per cent CO_2: 95 per cent air (Gladstone, Fildes and Richardson, 1935).

Minimal medium.

It was essentially that used by Gladstone (1937) and had the following composition:

1. KH_2PO_4 4·5 g., NH_4Cl 0·5 g., $(NH_4)_2SO_4$ 0·5 g., *ca* N/1 NaOH 24 ml. (to pH 7·4). Water 950 ml.
2. $MgSO_4.7H_2O$ 0·037 g. Water 10 ml.
3. Glucose 2·25 g. Water 20 ml.

Solutions 1 and 2 were autoclaved at 115° C. for 20 minutes in separate vessels, and 3 was Seitz filtered. Solutions 2 and 3 were added to 1 when cool and the mixture dispensed aseptically into tubes 11·0 cm. × 1·5 cm., using an automatic filler delivering 6 ml. to each tube. As necessary the medium was solidified with 1 per cent agar autoclaved separately. The customary precautions regarding purity of chemicals and cleanliness of glassware were observed throughout.

Complete medium.

Tryptic meat digest formed the basis of the complete medium. This was supplemented by the addition of 1 g. Difco yeast per litre and later further supplemented with purines and pyrimidines, each to a concentration of 50 μM.

Growth factors.

The following were held as stock solutions:

Glycine.	L (−) Cystine.	Aneurin.	Adenine.
Alanine.	Methionine.	Riboflavin.	Guanine.
Valine.	Aspartic acid.	Nicotinamide.	Xanthine
Leucine.	Glutamic acid.	Ca. pantothenate.	Hypoxanthine.
Isoleucine.	Lysine.	Pyridoxin.	Cytosine.
Norleucine	L (+) Arginine.	Choline chloride.	Thymine.
Proline.	L (−) Histidine.	Inositol.	Uracil.
L (−) Hydroxyproline.	Phenylalanine.	Biotin concentrate.	Ribose.
Serine.	Tyrosine.	Pimelic acid.	Haemin.
Threonine.	Tryptophan.	Para amino benzoic acid.	Glutamine.
			Oleic acid.
		Folic acid.	

Except where indicated the amino acids were racemic mixtures. The L-isomers were thrice recrystallized from suitable solvents.

EXPERIMENTAL AND RESULTS.

1. *Selection of ammonia assimilating strain of Ty2.*

A dried culture of Ty2 was four times selected from single colonies on TMA and confirmed as consistent with *Bact. typhosum* in its sugar reactions and agglutination by typhoid Vi, O and H antisera. Visible growth occurred in 66 hours following the inoculation of *ca.* 5×10^5 twice washed cells into minimal medium, both with and without added tryptophan (100 μM final concentration). Twelve serial transfers were made in minimal medium and the resulting culture four times selected from single colonies on minimal medium agar. The final culture grown on TMA resembled the original Ty2 in all respects. Loss of H antigen occurred when grown on minimal medium, as previously noted by Gladstone (1937). A considerable number of dried cultures, sufficient to outlast this study, were prepared and the strain, designated Ty22, used for all subsequent experiments.

2. *Induction of mutants.*

(a) *By X-irradiation.*—An apparatus manufactured by the Picker X-ray Corporation was employed and operated at 60 peak kilovolts with a tube current of 4 milliamperes. Five ml. of a suspension with 2×10^9 organisms per ml. was

contained in a sterile pyrex chamber 2·5 cm. diameter and 5 cm. deep in which a platinum stirrer, glass cooling coil and thermocouple were incorporated. The chamber was located vertically above the tube with its base 5 cm. from the target. Throughout irradiation the suspension was rapidly stirred and its temperature maintained between the limits of 6° C. and 12° C. by the circulation of ice-cooled water through the cooling coil. Samples were removed through a cotton-wool plugged side arm. Unirradiated suspensions retained their full viable counts throughout a period of 7 hours of rapid stirring with cooling in this chamber. 80 minutes' irradiation under our conditions reduced the viable count to *ca.* 2×10^4 organisms per ml.

(b) *By ultraviolet irradiation.*—Two ml. of suspension with 1×10^8 organisms per ml. was contained in a shallow glass vessel 3·5 cm. diameter located 50 cm. vertically below a Hanovia low pressure mercury discharge lamp delivering most of its radiation at a wavelength of 2657 Å. Exposure was conducted at room temperature for 2·5 minutes, by which time the viable count had been reduced to *ca.* 3×10^4 organisms per ml.

(c) *By nitrogen mustard.*—Tri-β-chlorethylamine hydrochloride (HN-3-HCl) was held as a 1 per cent (w/v) stock solution in distilled water. Of the suspension 1·98 ml. with 1×10^9 organisms per ml. in phosphate buffer pH 6·6 was incubated at 37° C. for 30 minutes. Of the stock solution of HN–3–HCl 0·02 ml. was then added and incubation continued for a further 30 minutes. This treatment reduced the viable count to *ca.* 5×10^6 organisms per ml.

3. *Enrichment of samples.*

(a) *By penicillin.*—Davis (1948, 1949*a*) obtained 80 per cent mutant cells among the survivors after subjecting irradiated suspensions of *E. coli* to a penicillin enrichment procedure. The enrichment depends on the selective bactericidal action of penicillin on growing cells, so that when a mixed population of mutant and non-mutant cells is incubated in minimal medium in the presence of penicillin, the non-mutant cells being capable of growth are eliminated, leaving the mutants unaffected. Lederberg and Zinder (1948) emphasized the necessity of modifying the technique to suit different organisms. In view of the number of possible variables in the technique it was not considered within the scope of this work to test all possible permutations and the technique finally adopted by us is possibly not optimal. We failed to obtain any mutants from X-irradiated samples subjected to the enrichment technique as described by Davis for *E. coli*. Experimentation with a mixed suspension consisting of equal numbers of Ty22 and of a mutant No. 479 (requiring a purine plus aneurin and isolated by the method of Beadle and Tatum) led to the development of a procedure giving a fifty-fold increase in the proportion of mutants to non-mutant cells among the survivors. However, when used on a mixture of 1000 Ty22 to 1 : 479 no mutants were recovered from 200 isolates. Applied to irradiated or mustard treated samples the procedure developed led to the isolation of a number of amino acid or vitamin-requiring mutants.

Samples after radiation or mustard treatment were grown overnight in complete medium. Of the complete medium culture 0·1 ml. was transferred to a complete medium slope, incubated 6 hours, emulsified and diluted in buffer and an inoculum of approximately one million organisms transferred to 4 ml. of

minimal medium from which the ammonium salts had been omitted. After 12 hours' incubation, ammonium salts to restore the concentration used in minimal medium, and penicillin to give a final concentration of 400 Oxford units per ml. were added (with the aid of an automatic device) and incubation continued for a further 6 hours. Penicillinase sufficient to inactivate residual penicillin was then added, the suspension diluted and plated on complete medium and the colonies, after 24 hours' incubation, examined for the presence of mutants. In many cases the suspension after penicillin treatment was again grown in complete medium and subjected to a second penicillin treatment, using the same procedure. Slight further enrichment was obtained by the double treatment. To avoid the repeated isolation of frequently occurring histidine or methionine requiring mutants these substances were added with the penicillin in later experiments.

A summary of the numbers of mutants obtained following penicillin enrichment procedures is given in Table I. Only those mutants having the same requirement derived from different samples are classified as independent mutants.

TABLE I.—*Mutants Obtained following Penicillin Enrichments.*

Mutagenic agent.	Type of enrichment.	Number of experiments.	Isolates tested.	Mutants obtained.	Independent mutants.
X-rays	Single	4	2316	18	13
	Double	4	2650	30	12
Ultraviolet	Single	8	3600	3	2
	Double	12	5366	61*	10
Nitrogen mustard	Single	4	1600	0	..
	Double	7	2768	6	5
	Totals	39	18,300	118	42

* Includes 52 mutants obtained from 200 isolates in one experiment. Of these 50 required histidine and 2 methionine.

(*b*) *By minimal supplementation.*—From the large number of cultures tested following treatment with penicillin no mutants requiring purines were obtained. Preliminary virulence tests with mutant 479 had shown these to be of interest and a method for their isolation, independent of penicillin treatment, was sought. Tests with known mixtures of Ty22 and the aneurin and purine-less mutant 479 showed that the colonies of each type could readily be distinguished when plated on minimal agar supplemented with limited amounts of purines sufficient to permit the mutants to develop into small colonies only. A method of detecting mutants by minimal supplementation was described by Davis (1949*b*). The medium employed by us was minimal agar plus 0·005 per cent (w/v) vitamin free casein hydrolysate and adenine, guanine, xanthine, hypoxanthine, cystosine and thymine each to 2·5 μM final concentration. Uracil was omitted, as it was found to be somewhat inhibiting. The constituents of minimal agar used in this method were of finest commercial grades, but not recrystallized.

Irradiated samples were grown overnight in complete medium supplemented with purines and pyrimidines, viable counts made and the appropriate dilution plated on supplemented minimal agar to give approximately 500 colonies per plate. After 48 hours' incubation all undersized colonies were isolated and tested

for mutants. The method was found to be simple and effective in eliminating a large proportion of non-exacting cultures prior to the final selection of mutants from mixed populations. It was only applied to X-irradiated samples. A summary of the numbers of mutants obtained in this way is given in Table II.

TABLE II.—*Mutants Obtained by Minimal Supplement Method following X-irradiation.* (*Dose rate* ca. 550 *roentgens per minute*).

Minutes irradiation.	Number of experiments.	Colonies examined.	Small colonies tested.	Mutants obtained.	Independent mutants.
0	1	5,800	12	0	0
40	1	12,000	31	5	5
60	8	98,600	212	39	23
80	7	125,800	301	43	23
100	5	22,850	68	3	3
Totals ..	21	259,250	612	90	54

4. *Selection of mutants.*

Initial attempts to isolate mutants from irradiated or mustard treated samples were made using the original method of Beadle and Tatum (1941), involving the transference of small inocula from complete medium agar to tubes of minimal medium and observing those which fail to grow on incubation. The method was very time-consuming and resulted in the selection of one mutant (No. 479) from 1755 cultures examined in this way. Two hundred and forty-one cultures tested subsequent to penicillin treatment yielded no mutants.

A considerable economy of time and materials was effected by adopting the following method, employed in the selection of all subsequent mutants. Penicillin treated samples were plated on complete medium agar to give 100 to 200 colonies per plate. Several hundreds of these taken at random were then transferred to correspondingly marked and numbered plates of minimal and complete medium, using the smallest inoculum possible just touched on the surface of the agar. Using a four-pointed platinum wire four colonies could be transferred to the pairs of plates between successive flamings. Two such wires used alternately permitted the transfer of 100 colonies to minimal and complete medium in 20 minutes. Incubation for 48 hours revealed non-exacting forms as robust colonies 2 to 3 mm. in diameter on the minimal agar whilst exacting forms remained as small film-like growths. The colonies on complete medium plates corresponding to the latter were then transferred to slopes for maintenance whilst their nutritional requirements were established. Small colonies isolated from minimal supplemented plates were selected for mutants in a similar manner. Fifty inocula were conveniently accommodated on each plate.

The exacting cultures obtained were confirmed as strains of *Bact. typhosum* firstly by their reaction to typhoid Type II E phage used in its critical test dilution for Ty22 and secondly by slide or tube agglutination with specific typhoid antisera.

5. *Determination of mutant requirements.*

An auxanographic method based on that described by Pontecorvo (1949) was used for the rapid determination of the growth factor requirements of mutants.

Plates of minimal agar were divided into a maximum of six sectors by 2 mm. wide channels, cut in the agar by means of a small " plough," and one well cut from the centre of each sector with a cork borer. Duplicate point inocula taken from mutant cultures on complete medium were placed radially around the wells, each of which contained a small volume of a different composite solution of growth factors. After 48 hours' incubation the solutions to which the mutants responded were in most cases obvious. The constituents of the active composite solutions were then tested individually or in combination by the same method to determine which were effective in promoting growth. All growth factor requirements determined by this plate method were confirmed by inoculation into tubes of minimal medium with and without the addition of the growth factors indicated.

Six different mutants could be conveniently spot-inoculated around each well, thus permitting the testing of six mutants against six different growth factors per plate. The channels were effective diffusion barriers preventing the added growth factors in one sector from masking the responses of inocula to those in adjacent sectors.

6. *Stability of mutants.*

A number of isolates failed to grow when first tested on minimal medium and were considered to be biochemical mutants. However, when subjected to the procedure used for the determination of mutant requirements they frequently showed full response to one factor, but evidenced instability by responding to a number of others on continued incubation. Such mutants, which presumably readily back-mutated to the non-exacting form, were considered unsuitable for further work and were discarded. Mutants responding to cystine or to glycine in the first instance were mostly of this very unstable type.

The quantitative responses of mutants to their respective growth factors added at various levels to tubes of minimal medium have been determined for most of those with different requirements to establish optimal levels for use in further work. On a number of occasions the instability of certain mutants was readily demonstrated during those determinations by the sudden recommencement of growth on prolonged incubation in those tubes containing insufficient growth factor addition to permit optimal growth of the mutant form.

Among the mutants retained by us those with requirements for cystine, glycine or proline appear particularly liable to reversion to the non-exacting condition. Similar reversion has been observed in those with arginine, phenylalanine and PAB deficiencies, whilst the remainder are apparently stable mutants or have low back mutation rates. Mutants with multiple deficiencies were found capable of loss of one or other or, more rarely, of all requirements, a property which made the establishment of the nutritional requirements of the original cultures a matter of some difficulty. In establishing the growth factor requirements of mutants the possibility of back mutation had to be constantly borne in mind and the growths obtained in tubes containing the supposed growth factors confirmed as those of mutants, and not of reverted organisms, by failure to grow on subculture into minimal medium.

7. *Types of mutants.*

Table III lists the different types of mutants obtained, their origin and the number of times independently isolated. As will be seen from Tables I and II,

the number of mutants isolated exceeds the number of independent mutants, since if more than one mutant with a particular requirement was isolated from any one sample, all such mutants were regarded as replicates derived from the original mutated cell by division and only one retained and included in Table III. Except where alternative requirements are indicated the mutants have not been observed to respond to the single addition of the other growth factors listed earlier.

DISCUSSION.

The very large increase in the proportion of mutant to non-mutant cells obtained by Davis (1949*a*) after penicillin treatment of irradiated suspensions

TABLE III.—*Types of Biochemical Mutants, How Obtained and Frequency of Independent Isolation.*

Growth factor requirements.	Number of independent isolations. Total.	By penicillin enrichment following treatment with: X-rays.	Ultraviolet.	HN_3.	By minimal supplement method after X-irradiation.
Glycine	5	2	1	..	2
Glycine or serine	1	..	..	..	1
Leucine	3	..	..	..	3
Valine + isoleucine	3	3	..	..	..
Proline	8	1	4	..	3
Cystine	10	3	..	..	7
Cystine or methionine	2	..	..	..	2
Methionine	16	5	3	..	8
Aspartic acid	3	1	1	1	..
Lysine	3	3	..	..	..
Arginine	5	2	..	..	3
Histidine	10	1	2	2	5
Phenylalanine	1	..	..	..	1
Tyrosine	1	..	..	..	1
Threonine	2	..	..	1	1
Aneurin	5	1	..	..	4
Nicotinamide	2	1	..	..	1
Pantothenate	1	1	..	..	..
Biotin	2	..	1	..	1
Para-amino benzoic acid	1	..	..	..	1
Any purine	1	..	..	..	1
Guanine or xanthine	1	..	..	..	1
Cytosine or uracil	3	..	..	..	3
Any purine + aneurin	3*	..	..	..	2
Valine + isoleucine + pantothenate	4	..	..	1	3
Not established	1	1	..	..	..
Totals	97	25	12	5	54

* Including mutant 479.

of *E. coli* was not achieved by us with *Bact. typhosum*. A pre-requisite for successful enrichment by this method is the establishment of conditions in a suspension in minimal medium in which mutant cells are theoretically unable to make a single division. In small scale experiments we have been able to demonstrate that Ty22 in the logarithmic phase of growth in minimal medium liberates into the medium sufficient growth factors to support considerable growth of all our different mutants with the exception of those requiring histidine, methionine, threonine, guanine, tyrosine or pantothenate. It is reasonable to assume, therefore, that in a mixed population of washed mutant and non-mutant cells syntrophic growth of many of the mutants could occur to a considerable extent, which, in the presence of penicillin, would lead to their elimination and reduce the efficiency of the enrichment. *E. coli* having a more perfectly balanced economy for growth in simple synthetic media, as evidenced by its higher growth rate than Ty22 in minimal medium, is probably less likely to support the development of mutants by growth factor over-production to such a large extent.

A number of interesting points arises from a survey of the types of mutants obtained. The high frequency with which mutants requiring histidine, methionine and cystine were selected is remarkable. Thus, of the 97 mutants obtained these three types account for over one-third, the remainder being composed of some 22 others. Had histidine and methionine requiring mutants not been specifically suppressed in the majority of penicillin enrichments their frequency would have been still higher. Several factors could contribute towards the higher frequency of isolation of particular mutants. Primarily this may result from a higher frequency of induction by virtue of the greater reactivity of particular genes with the mutagenic agents or their products, or, accepting the target theory for genetic mutation (Lea, 1947), from such genes occupying larger volumes so that they have a greater chance of the passage of a single ionizing particle through them for a given dose. The selection methods employed would favour those mutants having higher growth rates than the parent in complete medium, and those least able to grow syntrophically or more resistant to penicillin in enrichment treatments depending on its action.

It is most probable that among our mutants responding to cystine, to methionine or to histidine, blockage at different levels in the biosynthetic process of which these substances are terminal products are represented, and the frequency of a particular mutant may, to some extent, be indicative of the number of genetically controlled steps intervening between the presumed growth factor (e.g. histidine) and its nearest known precursor or precursors. With a fuller knowledge of the precursors involved in the synthesis of these three growth factors in *Bact. typhosum* and by a more comprehensive examination of the behaviour of our mutants to known precursors (e.g. sulphide in cystine synthesis) it is probable that these mutants could be assigned to a number of different types.

The relatively high frequency of mutants with a multiple requirement for valine plus isoleucine plus pantothenate is also notable. If biochemical mutation is a completely random process, other factors being equal it would be expected that multiple mutants would have requirements similar to those occurring with highest frequency among the simple mutants. However, no mutants requiring histidine plus methionine were obtained and no multiple mutants had a requirement for one or other of these amino acids plus another factor. Three valine plus isoleucine mutants were independently isolated and one pantothenate as

opposed to the four multiple mutants requiring all three substances. It is probable that the requirement for valine plus isoleucine is a similar phenomenon to that described by Bonner (1946) for *E. coli* mutants, where the blockage of a single gene controlled conversion of the keto acid analogue of isoleucine results in inhibition of valine synthesis. These multiple mutants have been observed to revert to the loss of a requirement for pantothenate or for valine plus isoleucine suggesting that two genes are involved in the mutation rather than one gene controlling the synthesis of two different substances. The three independent isolations of purine plus aneurin-requiring mutants is similarly notable.

The employment of the minimal supplement method effected a considerable economy of time and labour in the selection of biochemical mutants and yielded a variety of different types. The method was adopted mainly to obtain purine and pyrimidine mutants and the casein hydrolysate added primarily to induce more rapid growth. Supplementation with specific amino acids at known concentrations rather than with casein hydrolysate would probably assist the recovery of mutants requiring these substances as growth factors. However, with a casein hydrolysate addition, one in seven of the small colonies isolated was found to be exacting, whereas following penicillin enrichment an average of 155 were tested for each mutant obtained. Supplementation of complete medium with additional purines and pyrimidines possibly assisted the recovery of mutants with these requirements by the minimal supplement method, although all were found to grow abundantly on unsupplemented complete medium. Since no specific vitamin additions were made to minimal supplement plates it is presumed that the vitamin-requiring mutants isolated developed syntrophically or from vitamins present as impurities.

The mutants obtained provided a variety of material for the investigation of the effects of biochemical mutation on the virulence of *Bact. typhosum*, which will be reported in a subsequent paper.

SUMMARY.

A number of biochemical mutants of *Bact. typhosum* with requirements for amino acids, vitamins, purines or pyrimidines have been obtained following treatment with X-rays, ultraviolet light or nitrogen mustard, as a preliminary to a study of the effects of biochemical mutation on virulence.

The use of point inocula and solid media was found to facilitate the selection of biochemical mutants from mixed population and the determination of their growth factor requirements.

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