535. *Induced and Other Variations in Bacterial Cultures. Part VII.* The Stability of the Nutritional Requirements of the Strains isolated after Irradiation of* Bact. coli.

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Of the 78 strains of *Bact. coli* (Part V) initially showing nutritional requirements (amino-acids) out of 1092 tested after irradiation with ultra-violet light, two only have proved so far to be stable. The others were re-trained to grow in a synthetic medium of glucose, phosphate buffer, ammonium sulphate and magnesium sulphate by processes varying in complexity from a single subculture in presence of the requirement to successive subcultures through progressively simpler media. An almost continuous spectrum of stability is shown, and the return to maximum growth rate in the synthetic medium is also gradual and continuous. The hypotheses of reversed mutation and adaptive repair of damaged enzyme systems are compared.

It has been shown in Part V (*J.*, 1952, 2817) that of the 1092 mutant strains of *Bact. coli* isolated by various methods 78 were unable to grow in a standard synthetic medium (glucose, phosphate buffer, ammonium sulphate, magnesium sulphate) on inoculation from " Lemco " broth. They grew in this medium, however, when it was supplemented by amino-acids, and on the basis of their apparent nutritional requirements a provisional

* Part VI, preceding paper.

classification was drawn up. As in the previous study with *Bact. lactis aerogenes* (*J.*, 1951, 1169) the question now arises whether these requirements are of a permanent or transitory nature. Reference to Table 2 of Part V shows that when tested as soon as possible after isolation the nutritional requirements of 55 of these were so far from permanent that the strains grew in the standard synthetic medium after 1 subculture in an enriched medium.

The other 23 strains appeared to have more stable nutritional requirements. In Table 1 the number of relatively stable and unstable mutants in each of the groups of the classification is summarised.

TABLE 1.

Group in classification	Requirement	Number of strains	
		Relatively stable	Unstable
2	Asparagine	0	7
3	Asparagine–glutamic acid	20	27
4*a*	Any single amino-acid	0	8
4*b*	One specific amino-acid	2	12
4*c*	More than one amino-acid	0	1
4*d*	Complex	1	0
		23	55

A more detailed examination of the nutritional requirements of these 23 strains was then carried out. It will be convenient to treat the various groups in turn.

In group 3 there were 20 apparently stable strains, but when they were re-tested after resting for 5 weeks in " Lemco " broth at room temperature, 8 of them were found to have lost their requirement for asparagine and glutamic acid. The remaining 12 strains, however, would not grow in the standard synthetic medium and since they grew only to a very limited extent when this was supplemented with asparagine and glutamic acid (total population 5—10 $\times$ 10^6 bacteria per ml., compared with a normal value of about 3—4 $\times$ 10^8) even after several subcultures in it, it seemed possible that the actual growth requirement was an impurity in either the asparagine or the glutamic acid. The strains were therefore re-tested in the standard synthetic medium supplemented by various amino-acids. The results are given in Table 2. In the presence of the nutrient named in this table a normal stationary population was obtained and thus, although asparagine and

TABLE 2.

Supplement	No. of strains	No. of strains which grew in standard synth. medium after 1 subculture in presence of supplement
Arginine	2	0
Leucine	2	1
Serine	4	2
Valine	1	0
Leucine or valine	1	1
Serine or lysine	2	0
	12	4

glutamic acid supported some growth, they were not the factors which resulted in optimal growth.

Four of these strains grew in the standard synthetic medium after 1 subculture in the presence of the new supplement. The other 8 strains were re-tested again after remaining for a further 3 weeks in broth at room temperature and it was found that 1 strain which had previously required leucine was now able to grow without it. Further experiments were carried out on the remaining 7 strains. They were subcultured 4 times in the standard synthetic medium supplemented by all the amino-acids in admixture, and then were inoculated into the asparagine–glutamic acid medium. Three of the 7 strains grew in this medium, and after 3 subcultures in it were able to grow in the unsupplemented standard synthetic medium. These 3 strains had previously required valine, arginine, and serine or lysine, respectively.

A further test of the stability of the remaining 4 strains in this group was next carried out. They were subcultured in the standard synthetic medium in the presence of their

growth requirement, and the concentration of the latter was gradually reduced to zero. This experiment is summarised in Table 3.

TABLE 3.

Strain no.	Amino-acid	Stationary population (millions per ml.), n_s						
	Subculture no.:	1	2	3	4	5	6	7
P 34/1061	Arginine	199	204	222	196	185	185	150
1228	Serine	150	110	70	123	150	150	136
1285	,,	140	94	59	64	30	30	30
1294	,,	137	94	56	50	233	233	110

	Subculture no.:	8		9		10	
		Lag (hrs.)	n_s	Lag (hrs.)	n_s	Lag (hrs.)	n_s
P 34/1061		∞	—	—	—	—	—
1228		<15	140	<15	150	<15	180
1285		∞	—	—	—	—	—
1294		<15	140	36	143	<15	180

Subculture no.	1	2	3	4—7	8—10
Concn. of amino-acid (mg. per 26 ml. of medium)	5	3	2	1	0

Inoculum during serial subculture = 0·1 ml.
Lag ∞ = no growth during 14 days.

By this method the number of stable mutants in this group had now been reduced to 2. Mutant No. P 34/1061, which requires arginine, will also grow in the presence of citrulline but not ornithine. The interrelationship of ornithine and citrulline in the biosynthesis of arginine by *Neurospora* has been reported by Srb and Horowitz (*J. Biol. Chem.*, 1944, **154**, 129).

Group 4*b* of the classification (one specific amino-acid as requirement) contained 14 strains, 12 of which were unstable (see Part V, Table 3). The other 2 strains, like some of the members of group 3, regained the ability to grow in the standard synthetic medium after a period of storage in "Lemco" broth. It has been observed previously in these laboratories that damaged bacteria recover, in some cases, when stored in broth. The simplest explanation is that lysis and re-synthesis occur during the storage.

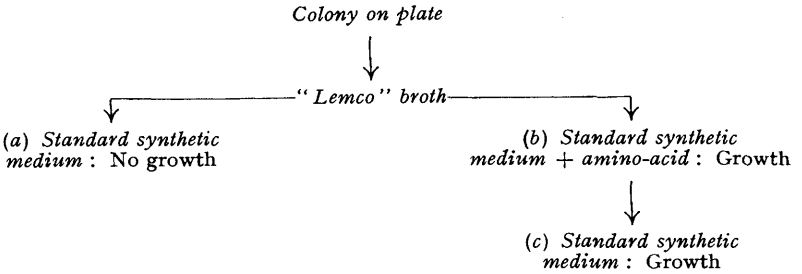
The growth requirements of the mutant in group 4*d* were complex, but after serial subculture in the asparagine–glutamic acid medium containing casein hydrolysate at a concentration which was gradually reduced to zero, it was able to grow in the asparagine–glutamic acid medium though not in the standard synthetic medium.

It can thus be said that of the 78 strains with an apparent nutritional requirement, this appears, so far, to be permanent in only 2 cases (or 3 cases if we include the strain whose requirement has been reduced from casein hydrolysate to asparagine–glutamic acid). There were, however, various degrees of instability. Some of the mutants, when tested soon after isolation, regained the ability to grow in the absence of the amino-acid after 1 subculture in its presence; with others a period of storage in a complete medium had also to be allowed. Some strains which did not yield to either of these two methods of treatment were trained to grow in the unsupplemented standard synthetic medium after subculture in a rich medium (all the amino-acids) followed by growth in the asparagine–glutamic acid medium. Finally, even this latter treatment did not prove sufficient with 2 strains. They were eventually trained by serial subculture in the standard synthetic medium containing diminishing amounts of their own initial growth requirement.

The mechanism by which this recovery takes place is of interest. The general behaviour of these unstable strains is very suggestive of that of the strains, which apparently required asparagine–glutamic acid (group 1), isolated from the earlier experiments on *Bact. lactis aerogenes* and described in the preceding papers in this series (*J.*, 1951, 1157, etc.). The recovery of these strains was shown probably to be adaptive, but in the present examples there still remains the possibility that reverse mutation may be responsible, and the problem of mutation or adaptation must again be considered.

First there were the 55 strains which were unstable when tested soon after isolation. These strains would always have been isolated as mutants even by the most rigid criteria

since they did not grow in the standard synthetic medium in the absence of the growth requirement until they had grown once in its presence. The treatment of these strains may be summarised thus :



When first grown in the absence of the growth requirement 38 of these strains had lags of less than 1 day (this is of the same order as that expected from a normal organism with an inoculum of 1 loop) ; with 8 strains the lag was 1—2 days ; with 4 strains 2—3 days and with 5 strains greater than 3 days. In the second subculture the lag was usually of normal duration and in the third subculture it was normal with all the strains (Part V, Table 2).

At first sight these results might suggest that a discontinuous mutation (reversion) had taken place at some stage between (*b*) and (*c*) (see diagram) and resulted in normal organisms. To investigate this, 9 of these strains were serially subcultured in the standard synthetic medium until their growth rates had reached the normal value. Mean generation times were determined at intervals during the training process. Results are given in Table 4. The behaviour is very similar to that found previously for mutants of *Bact. lactis aerogenes* with which, after a detailed investigation, it was shown that adaptation was the most economical hypothesis to explain the observed behaviour (*J.*, 1951, 1157). The fact that the growth rates were not normal during the first subculture at which growth would occur in the unsupplemented standard synthetic medium is evidence at least that a single reverse mutation is not the mechanism by which the strains recover.

The strains which, although unable to grow in the standard synthetic medium after the treatment outlined in the preceding diagram, grew in it after a more elaborate treatment [(i) storage in broth ; or (ii) growth in a mixture of all the amino-acids followed by growth in asparagine–glutamate medium ; or (iii) growth in the standard synthetic medium containing diminishing amounts of the growth requirement], showed the same pattern of lags in the standard synthetic medium as the previous group. When the growth rates, during serial subculture, of some of these strains were examined, the behaviour proved to be identical with that of the strains in the first group (Table 4).

Whether the 3 strains, which, so far, appear to have more stable nutritional requirements, have sustained damage of a discrete genetic nature or whether they represent the extremes in a continuously graded series of changes in the molecular pattern of the cell substance, cannot be stated with certainty at present. But when the other results are taken into consideration the latter view seems much the more probable. It may be that, after a still longer period of storage in broth, these 3 more stable strains will become unstable. One of them, which originally required some factor or factors in casein hydrolysate, has been trained to grow in asparagine–glutamic acid medium. The other 2 require arginine and serine respectively.

Of 78 of these strains obtained by ultra-violet irradiation of *Bact. coli*, 7 grew in the presence of asparagine and 35 in asparagine–glutamic acid medium. It is of interest to compare these results with those reported earlier for *Bact. lactis aerogenes*. With the latter organism no mutants were isolated which did not grow readily in asparagine–glutamic acid medium. Besides the 42 strains of *Bact. coli* which grew in the presence of asparagine or asparagine–glutamic acid, 1 strain grew in the presence of any one of the commoner amino-acids, 1 strain in any of 8 amino-acids, 9 strains in either of 2 amino-acids, and 23 strains in 1 specific amino-acid. One strain appeared to require a mixture of all the amino-acids, and one would only grow in the presence of casein hydrolysate. The commoner require-

ments of the 23 strains which grew in the standard synthetic medium when supplemented with 1 specific amino-acid were: tryptophan (11 strains), serine (4 strains), arginine (3 strains), and leucine (3 strains).

The principal object of these experiments was the systematic investigation of the behaviour of the more exacting *Bact. coli* strain for comparison with the less exacting

TABLE 4. *Training of irradiated strains.*

Strain no.	Subculture no. in standard synth. medium	M.g.t. (min.)	Method of training †	Group in classification
Parent	—	40	—	—
P34/1	2	55	1	2
	25	39		
/2	25	45	1	3
	53	40		
/136	10	46	1	2
	37	41		
/190	6	51	1	4*b*
	33	40		
/1084	2	65/43 *	1	4*b*
	4	38		
/1091	4	53	1	4*a*
	7	39		
/1107	3	51/56 *	1	4*b*
	7	50		
	16	38		
/1108	2	48	1	3
	11	40		
/1143	4	52	3	4*b*
	7	41		
/1256	4	74		4*b*
	21	39		
/1258	4	70		4*b*
	8	52		
	12	48		
	19	146/51 *		
	25	46		
	33	39		
/1294	5	140		4*b*
	9	55		
	15	55		
	21	53		
	29	40		

* Composite growth curve.

† *Methods of training*:

1, One subculture in growth requirement, then serial subculture in standard synthetic medium.
2, Storage in " Lemco " broth, then serial subculture as in 1.
3, 4 Subcultures in presence of all amino-acids, followed by 3 subcultures in asparagine–glutamic acid, then serial subculture as in 1.
4, Serial subculture in standard synthetic medium containing diminishing concentrations of growth requirement. (The subculture numbers in the table denote the number of subcultures after the concentration of the growth requirement had reached zero, *i.e.*, no. of subcultures after no. 7 in Table 3.)

Bact. lactis aerogenes. They were not designed to yield crucial distinctions between the view, on the one hand, that instability of mutants and recovery of power to grow in unsupplemented media are due to reverse mutations of a discontinuous and random kind, and, on the other, the apparently opposed view that quantitative damage to enzyme systems occurs on irradiation and is repaired during the use of these damaged systems in actual growth.

General considerations of probability favour the second view. The degree to which the cells become exacting on irradiation varies according to an almost continuous spectrum, and the pattern of recovery is one of continuous repair rather than of the emergence at any stage of a qualitatively different organism. The number and variety of genes which would be involved in these processes might be said to render the distinction between the two formally different views very indefinite. Furthermore, as regards the frequently alleged fortuitous character of the repair process, the whole technique of re-training strongly

suggests that opportunity has to be provided for the exercising in easy stages of the more seriously impaired enzyme mechanism, and that only in the rarest cases has an enzyme mechanism been completely extirpated (without death of the cell).

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