262. *Induced and Other Variations in Bacterial Cultures. Part IV. Restoration of Normal Biochemical Reactions in Fermentative Mutants of* Bact. lactis aerogenes.

By A. C. R. Dean and Sir Cyril Hinshelwood.

The restoration of normal fermentative reactions in mutants of *Bact. lactis aerogenes* is subjected to a quantitative study in the light of theories of back mutation and of repair to damaged enzyme systems.

It has been mentioned previously (Part II, *J.*, 1951, 1159) that, after the training to the normal growth rate, the biochemical reactions of the irradiated *Bact. lactis aerogenes* strains were normal. The manner in which the cells regain their ability to ferment various substrates should provide further evidence relevant to the question of mutation or adaptation. If a single reverse mutation is responsible for the recovery, all the lost functions should re-appear simultaneously, while with adaptation graded recovery is quite likely. Graded recovery would also occur if a whole series of minor mutations was involved, but in this case the individual biochemical characters should at least re-appear with full quantitative intensity (unless very special hypotheses about mutual influences of characters are invented).

Strain No. 14 was inoculated from broth into the asparagine–glutamic acid medium, and from the latter was serially sub-cultured in the synthetic medium twice daily until trained. Sugar fermentation tests were carried out at every fourth sub-culture, with the wide range of sugars described previously. Control experiments with a normal strain were set up with every experiment. A normal strain of *Bact. lactis aerogenes* ferments all the sugars, except D-arabinose, in 24 hours at 37°, the arabinose requiring 48 hours. The usual American practice (Prescott, Winslow, and McGrady, " Water Bacteriology," Wiley, New York, 1946, p. 202) allows 48 $\pm$ 3 hours as a time limit for the coliform group, and in order to ensure a wide margin of safety, the fermentation tests were kept for one week, and were read at daily intervals.

Over the first 68 sub-cultures the only difference in the results with increasing time of test was a tendency for an acid reaction to become alkaline, and hence only the results after one week were recorded. If an acid reaction was given at first, even if it subsequently became alkaline, it is reported as acid (Table I). From sub-culture 72 to 117 detailed results are necessary, and are shown in Table II.

TABLE I.

Fermentation reactions during serial sub-culture of strain No. 14 in the normal synthetic medium. Results after one week.

Sugar.	A/G †	Sub-cultures in normal synthetic medium.																
		1	4	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64	68
D-Arabinose	—	A	—	—	—	—	A	A	—	A	—	—	A	—	—	A	A	—	—	—
Xylose	—	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Raffinose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glucose	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Mannose	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Galactose	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Sucrose	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A	—	—	—
Maltose	A	—	A	—	A	A	A	—	—	A	A	A	A	A	—	A	A	A	—	—
Lactose	—	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	—	A	A
Cellobiose	A	A	A	A	A	A	A	A	A	A	A	A	A	A	—	—	—	A	—	—
Melibiose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inositol	—	A	A	A	—	—	A	A	A	—	—	A	A	—	—	A	A	—	—	—
Sorbitol	A	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Rhamnose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
M.g.t.	—	—	51	48	46	48	55	59 * / 47	40	62 * / 40	51	43	30 * / 46	38	37 * / 41	37 * / 41	44	42	42	52 * / 33

* Composite growth curve. —, No acid, no gas.
A, Acid. † Asparagine–glutamic acid medium.

The recovery of the fermentative abilities is gradual, and for more than half of the training period acid was produced from some sugars whilst with others there was no reaction. Then the ability to produce gas was recovered gradually. It took place first with sucrose as the substrate (sub-culture No. 72); the amount produced was small, and 5 days' incubation was necessary. This property was then lost, and re-appeared at sub-culture No. 84, disappeared again, and was not apparent again until almost the end of training process. Meanwhile gas production appeared with mannose (sub-culture No. 80), xylose, maltose, lactose, and cellobiose (sub-culture No. 93), and with glucose (sub-culture No. 101), and in these instances the same fading and re-appearance took place.

It is interesting that glucose and xylose were the only substrates from which acid was produced in every test.

When sub-culture No. 117 was fully trained, acid and gas were produced from all the sugars, except arabinose, within 24 hours, and from arabinose within 48 hours. This is the behaviour of a normal strain of *Bact. lactis aerogenes*, and the re-trained strain retained this behaviour after storage for one month in broth. The effect of any variation in the media used is ruled out by the fact that control tests with a normal strain carried out concurrently with each set of sugar reactions always yielded acid and gas from all the sugars except arabinose in 24 hours, and from arabinose in 48 hours.

In seeking possible explanations for the behaviour encountered in these tests it is necessary to have a clear picture of the operations involved in the tests. They may be summarised as follows:

growth in synthetic medium ⟶ growth in peptone water ⟶ growth in peptone water in the presence of the various sugars

The first explanation to be considered is that of a single genetic mutation (reversion), *i.e.*, at some stage in the growth processes a normal organism is thrown off. If such a reversion had

Footnotes to Table II.

* 8 and 9 days same as 7 days. —, No acid, no gas. A, Acid, no gas.
$\frac{A}{sg}$, Acid, small volume of gas. AG, Acid, normal volume of gas.
† 6 days and 7 days same as 5 days.

TABLE II.

Fermentation reactions during serial sub-culture of strain No. 14 in the normal synthetic medium. Detailed results.

Sub-culture number in normal synthetic medium.

Sugar.	No. 72.							No. 76.							No. 80.							No. 84.						
D-Arabinose	—	—	—	—	—	—	—	—	—	—	A	A	—	—	—	—	—	—	A	A	—	—	—	—	—	—	—	—	—
Xylose	—	A	A	A	A	—	—	—	—	A	A	—	—	—	A	A	A	A	A	—	—	—	—	A	A	A	—	—	—
Raffinose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Glucose	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	—	—
Mannose	A	A	A	A	A	A	A	A	A	A	A	A	A	A	—	—	A	A	A	A	A/sg	—	A	A	A	A/sg	A/sg	A/sg	
Galactose	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	—	A	A	A	A	A	A	A	
Sucrose	—	A	A	A	A/sg	A/sg	A/sg	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A	A/sg	A/sg	A/sg	
Maltose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A	A	A	A	A	—	—	
Lactose	—	A	A	A	—	—	—	—	A	A	A	A	A	A	A	A	A	A	A	—	—	A	A	A	A	A	A	A	
Cellobiose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Melibiose	—	—	—	—	—	—	—	A	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Inositol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Sorbitol	—	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Rhamnose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Time (days)	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7*	1	2	3	4	5	6	7	
M.g.t. (mins.)				52							52							42							46				

Sugar.	No. 89.							No. 93.							No. 97.							No. 101.						
D-Arabinose	—	—	—	—	—	—	—	—	A	A	A	A	A	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Xylose	—	A	A	A	A	A	—	A	A	A/sg	A/sg	A/sg	A/sg	—	—	—	A	—	—	—	—	—	—	A	A	A	A	—	
Raffinose	A	A	A	A	A	A	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Glucose	A	A	A	A	A	A	—	A	A	A	A	A	A	—	A	A	A	A	A	A	A	A	A	A	A	A	A	A/sg	
Mannose	—	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	—	—	—	—	—	A	A	A	A	A	A	A/sg	
Galactose	A	A	A	A	A	—	A	—	A	A	A	A	A	A	—	—	—	—	—	—	—	A	A	A	A	A	A	—	
Sucrose	—	—	—	—	—	A	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A	A	A	A	A	A	—	
Maltose	—	—	—	—	—	—	—	A	AG	AG	AG	AG	AG	AG	—	—	—	—	—	—	—	—	A	A	A	—	—	—	
Lactose	—	A	—	—	—	—	—	—	A	A/sg	A/sg	A/sg	A/sg	A/sg	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Cellobiose	—	—	—	—	—	—	—	A	AG	AG	AG	AG	AG	AG	—	—	—	—	—	—	—	A	A	A	A	A	A	A/sg	
Melibiose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Inositol	—	—	—	—	—	—	—	—	A	A	A	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Sorbitol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Rhamnose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A	—	—	—	
Time (days)	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7*	
M.g.t. (mins.)				33/54							36							44							41				

Sugar.	No. 105.							No. 109.					No. 117.		Normal strain.	
D-Arabinose	—	—	—	—	—	—	—	—	—	—	—	—	—	AG	—	AG
Xylose	—	A	A	A	A	A	A	A	A	A	A	A/sg	AG	—	AG	—
Raffinose	—	—	—	—	—	—	—	—	—	—	—	—	AG	—	AG	—
Glucose	A	A	A	A	A	A	A/sg	A	A	A	A	A/sg	AG	—	AG	—
Mannose	—	A	A	A	A	A	A/sg	A	A	A	A	A/sg	AG	—	AG	—
Galactose	A	A	A	A	A	A	—	A	A	A	A	A/sg	AG	—	AG	—
Sucrose	—	A	A	A	A	A	A/sg	A	A	A	A	A/sg	AG	—	AG	—
Maltose	—	A	A	A	A	A	—	A	A	A	A	A/sg	AG	—	AG	—
Lactose	—	A	A	A	A	A	—	A	A	A	A	A/sg	AG	—	AG	—
Cellobiose	—	A	A	A	A	A	—	A	A	A	A	A/sg	AG	—	AG	—
Melibiose	—	—	—	—	—	—	—	—	—	—	—	—	AG	—	AG	—
Inositol	—	—	—	—	—	—	—	—	—	—	—	—	AG	—	AG	—
Sorbitol	—	—	—	—	—	—	—	—	—	—	—	—	AG	—	AG	—
Rhamnose	—	—	—	—	—	—	—	—	—	—	—	—	AG	—	AG	—
Time (days)	1	2	3	4	5	6	7*	1	2	3	4	5†	1	2	1	2
M.g.t. (mins.)				42						38			31		32	

Footnotes at foot of opposite page.

taken place during the serial sub-cultures in the synthetic medium, the mean generation time and fermentation reactions in all the succeeding sub-cultures would have been normal. This could only have taken place between sub-cultures 109 and 117, and is very unlikely in view of the gradual recovery of the sugar reactions. The possibility that reversion may have taken place during the actual sugar tests themselves must also be considered. With any one sugar four possible results must be explained, *viz.*, no reaction, acid, acid with a small volume of gas, and the normal reaction. It would be necessary to postulate one mutation resulting in the ability to produce acid, *i.e.*, resulting in the appearance of the enzyme or enzymes necessary for transforming the sugar into hexose phosphate. Since glucose was degraded to acid in every test, it may be assumed that the enzymes necessary for the conversion of hexose phosphate into acid are already present in the cell. With arabinose, rhamnose, sorbitol, and inositol, the route may not be *via* hexose phosphate; in these cases the assumption would be that the mutation results in the appearance of an enzyme or enzymes necessary for the transformation of each substrate into acid. Another mutation resulting in the formation of gas would also need to be assumed, and it is difficult to see how this would account for the production of the small volume of gas obtained during the training process, unless the mutation took place when almost all the nitrogen in the culture medium had been used up, limiting the amount of enzyme that could be formed.

Further difficulties are encountered with the simple form of the mutation hypothesis when it is considered that the mutation resulting in acid production would have to take place very frequently, whilst that resulting in the production of gas would be of much rarer occurrence. In fact, the latter mutation would not have occurred at all during the first half of the training period, and fairly infrequently during the second part of the process, until the penultimate series of tests, in which the frequency would have increased greatly. In this penultimate stage, two types of response only were obtained. With 6 sugars no reaction took place, *i.e.*, no mutation could have occurred; from the remaining 8 sugars, acid and a small amount of gas were produced, *i.e.*, both mutations must have taken place together, and in all of them the mutation responsible for gas production must have taken place when almost all the nitrogen in the culture medium had been used up. Such a combination of events seems improbable.

The gradual recovery of fermentative abilities during training tends, by itself, to favour the alternative theory of adaptation as an explanation for the observed behaviour. This would postulate that, as the strain is serially sub-cultured in the normal synthetic medium, damaged enzyme systems are repaired and expanded until the growth rate and biochemical properties become normal. Incidentally, the postulate would include the hypothesis of Sevag (*Adv. in Enzymology*, 1946, **6**, 83), who believes that in some cases the recovery of irradiated cells can be explained on the basis of the return to the native state of certain proteins, which have been denatured, deformed, or distorted by the irradiation.

Whatever might be postulated about multiple mutations to explain graded reversion, one fact which would remain difficult to account for is the sort of time relation exemplified by the behaviour at sub-cultures 105, 109, and 117 (Table II). With mannose, for example, at 105 small amounts of gas appear after 7 days of test, at 109 they are evident after 5 days only, while at 117 not only does gas appear in full amount, but within one day. This certainly has the appearance of a quantitative development rather than of a qualitative discontinuity in character.

Although the conditions for carrying out the sugar tests were standardized as far as possible, it is evident that the cells from the various sub-cultures in the normal synthetic medium would not all be of the same biological age, and hence the enzymes would have decayed to varying degrees. This would result in different lags in the peptone water, and since the cells were inoculated into the sugar media after a definite time in the former, the properties of the various enzymes in the cells used in the different experiments would not be the same. Such behaviour would explain the production of acid in one experiment and its absence in subsequent experiments with the same sugar. Since the cells were serially sub-cultured in the glucose medium, it is not surprising that acid was invariably produced from it in the sugar tests. The enzymes in the cells would be better adapted to glucose than to any other sugar, and it is believed that in the aerobic and anaerobic disruption of glucose the same enzymes are concerned with part of the process, namely the conversion of glucose into pyruvic acid. Sevag (*ibid.*, p. 77) has suggested that hexokinase is responsible for converting glucose, mannose, and galactose into hexose phosphates in yeasts. It is interesting to note that acid was produced from galactose in all but one test, and from mannose in all but two tests. Probably other factors are also involved besides hexokinase in the fermentation of these two sugars.

Genetic Recombination.—Genetic recombination has been stated to occur by Tatum and

Lederberg (*J. Bact.*, 1947, **53**, 673) in a strain of *Bact. coli*. A synthetic agar medium was used as a selective sieve to isolate occasional recombinants which appeared in mixed cultures of complementary biochemical mutants. Later experiments introduced other factors, such as fermentation and virus-resistance, and these factors were found to segregate in a manner which suggested linkage.

A crossing experiment was carried out with two strains with abnormal biochemical characters One of them, No. 14, had been isolated by ultra-violet irradiation of *Bact. lactis aerogenes*, and was " sucrose-acid (no gas)," and " xylose-negative "; the other, F 23/5, isolated from an experiment with Fenton's reagent, was " sucrose-negative " and " xylose-acid (no gas)." 10 Ml. of broth were inoculated with one loopful of each of the strains and incubated for 3 weeks, after which a dilution was plated out on a complete agar, and 10 colonies were selected and tested for sucrose and xylose fermentation. Controls were put on at the same time for both of the strains. Results appear in Table III.

TABLE III.

" Crossing " experiment between strain No. 14 *and strain* F 23/5.

	No. 14.		F 23/5.		Cross.	
	Suc.	Xyl.	Suc.	Xyl.	Suc.	Xyl.
24 *Hours.*						
1	A	—	—	—	—	—
2	A	—	—	—	—	—
3	A	—	—	—	—	—
4	A	—	A	—	—	—
5	A	—	—	—	A	—
6	A	—	—	—	—	A
7	A	—	—	—	A	—
8	A	—	—	—	—	A
9	A	—	A	—	—	A
10	A	—	—	—	—	A
72 *Hours.*						
1	A	—	—	A *	—	A
2	A	—	—	A	—	A
3	A	—	*A* *	*A*	—	A
4	A	—	*A*	*A*	—	A
5	A	—	*A*	*A*	*A*	*A*
6	A	—	—	A	—	A
7	A	—	—	A	*A*	*A*
8	A	—	—	A	—	A
9	A	—	*A*	*A* *	—	A
10	A	—	—	A	—	A

	No. 14.		F 23/5.		Cross	
	Suc.	Xyl.	Suc.	Xyl.	Suc.	Xyl.
48 *Hours.*						
1	A	—	—	—	—	A
2	A	—	—	A	—	A
3	A	—	—	—	—	A
4	A	—	A	—	—	A
5	A	—	A	—	*A*	*A*
6	A	—	—	A	—	A
7	A	—	—	A	*A*	*A*
8	A	—	—	—	—	A
9	A	—	A	—	—	A
10	A	—	—	—	—	A

Parent cultures.

	Sucrose.	Xylose
No. 14	A	—
F 23/5	—	A

* Weak reaction.

Note.—*A* (italic) indicates result of crossing.

Even if rare cell conjugations leading to recombinations of genes occurred, it is obvious that the chance of isolating the relevant progeny might be negligibly small in the present case where no screening process is available. Nevertheless the sugar tests actually gave results which might have been formally interpreted as indicating a crossing of the two strains. Nos. 5 and 7 at 48 hours show the combination AA instead of A– or –A. But at 72 hours four of the controls had also developed the characters of crosses, so that the apparent evidence of recombination is revealed as completely illusory. One point which may perhaps be emphasised here is that had the test been cut off at 48 hours apparently controlled evidence of crossing would have been reported. The importance of the quantitative factor in test times is here revealed once again. It is not always observed, though Lindegren emphasises it throughout his book (" The Yeast Cell," Educational Publ., Saint Louis, 1949).

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