Summary

A statistical study founded on 300 elements (*Bacillus cereus alesti*, with a test: *Bombyx mori* larvae. Lepidoptera). Capacity for development and virulence respond in a parallel manner to ultraviolet radiation 2537 A; but the inhibition of virulence is more rapid than that of development. Irradiation for 4 to 5 hours gives avirulent elements which are, however, capable of development.

Studies on a Non-Heritable Physiological Modification in *Aerobacter aerogenes*

Heritable and non-heritable modifications arise in the ability of the bacterium *Aerobacter aerogenes* to grow in media containing *slow-adaptive substrates* as sole carbon sources[1]. These adaptive substrates include certain Krebs cycle compounds, such as citrate, alpha-ketoglutarate, succinate, fumarate, and acetate. This communication deals with some studies made on the induction of the non-heritable changes. In these studies populations were grown in a synthetic medium containing inorganic salts[2] plus d-glucose or an equivalent concentration (on a carbon-for-carbon basis) of one of the adaptive substrates. The number of cells capable of growing on a specific carbon source was determined by plating a suitable dilution of the culture in appropriate media containing 2% by weight of washed agar[3]. Populations to be irradiated were diluted in physiological saline solution to a density of about 500 cells per ml. From 4 to 8 ml of the diluted culture were irradiated with constant stirring in open beakers by a low pressure mercury arc (steri-lamp), about 90% of whose energy output is at a wavelength of 254 mμ.

The following observations can be made by plating cells from a liquid glucose culture in two different agar media, one containing glucose and the other citrate: (1) if the plated cells are in their logarithmic phase of growth or if their growth has been stopped due to glucose-exhaustion of the liquid culture, then the number of colonies formed in glucose and citrate agar is the same; (2) if the growth of the plated cells has been stopped due to the production of low pH in an unaerated culture containing an excess of glucose, then the number of colonies formed in glucose agar is greater than that formed in citrate agar, and the magnitude of the discrepancy between the two colony counts is a function of the length of time the cells are exposed to the culture conditions existing in the stationary phase (see Fig. 1); (3) if logarithmic phase cells are irradiated with ultraviolet prior to plating, then a similar difference in colony counts is found, which, however, depends upon the dose of ultraviolet employed (see Fig. 2). From these observations it is clear that specific culture conditions and ultraviolet irradiation can both induce heterogeneity in populations of *Aerobacter aerogenes* with respect to the ability to grow in media containing citrate as sole carbon source. In the case of stationary phase cultures, it has been found that the induction of heterogeneity is specific for the class of slow-adaptive substrates[4]. In the case of irradiated cultures the ability to grow in succinate, as well as citrate media is known to be affected (Fig. 2).

[1] A. W. RAVIN, J. Gen. Microbiol. (in press); (Ph. D. thesis, Columbia University, 1951).

[2] A. W. RAVIN, J. Gen. Microbiol. (in press).

[3] F. J. RYAN, Methods Med. Research *3*, 51 (1950).

[4] A. W. RAVIN (Ph. D. thesis, Columbia University, 1951).

The cells that have thus lost their ability to grow in media containing slow-adaptive substrates as sole carbon sources do not inherit their physiological alteration. This is in contradistinction to the genetic modification that is observed in certain types of noncitrate-utilizing mutants that appear spontaneously at a low frequency in glucose cultures of *Aerobacter aerogenes*[1]. Presumptive evidence of the non-heritable nature of the modification was obtained in tests of samples of glucose agar colonies derived from heterogeneous cultures, stationary phase or irradiated. Although the number of colonies tested was always far in excess of that necessary to include the colonies developing from originally non-citrate-utilizing (or non-succinate-utilizing) cells, every colony tested was capable of growth in media containing citrate (or succinate) as the sole carbon source. Proof was furnished by experiments in which heterogeneous stationary phase populations were inoculated into fresh glucose medium. It was observed that such populations become homogeneous again in such a brief period of time that selection of the unaffected cells cannot be invoked as the homogeneity restoring mechanism. For example, Table I furnishes data from an experiment in which a heterogeneous population was washed and aliquots were then inoculated into unaerated media, which either contained glucose or lacked any carbon source. The population became completely homogeneous after having been allowed to grow four hours in the presence of glucose. Since *Aerobacter aerogenes* has a generation time of 43 minutes in unaerated glucose medium[1] and, therefore, can increase at most fourfold in 1½ hours and about 32-fold in 4 hours, then the observed increases in the citrate- and succinate-utilizing components of the population can only be explained by some process wherein non-citrate-(or non-succinate-)utilizing cells are reconverted into utilizers. The appropriate calculations based on this experiment show that the probability of such a reconversion is from 0·53 to 0·86 within 1½ hours, depending on whether an exogenous carbon source is present. The presence of a carbon source apparently speeds and allows the completion of the process. Other experiments performed with an auxotrophic (prolineless) mutant indicate that the restoration of homogeneity is correlated with the active metabolism of the initially heterogeneous population, since the absence of the required nutrient (proline) in the medium acted to considerably reduce the rate of the restorative process.

Table I. Restoration of Homogeneity in a Heterogeneous Culture

Aliquots from a heterogeneous stationary phase culture were inoculated into media under the following conditions, and the restoration of homogeneity to the populations was followed by platings in glucose (G), citrate (C), and succinate (S) agars. Values shown (except for ratios) are expressed in cells $\times 10^5$ per ml. The ratios indicate the degree of heterogeneity in the culture at the time of sampling; ratios greater than 1·5 are significant.

Medium	Time (hours)	G	C	S	G:C	G:S
Salts minus glucose	0	51·1	9·7	0·13	5·3	389·7
	1½	84·0	52·0	–	1·6	–
	4	75·0	39·0	9·0	1·9	8·3
Salts plus glucose	0	51·1	9·7	0·13	5·3	389·7
	1½	88·0	60·0	–	1·5	–
	4	160·0	180·0	173·0	0·9	0·9

[1] A. W. RAVIN, J. Gen. Microbiol. (in press).

If homogeneous populations are inoculated into the sterile-filtered media of heterogeneous stationary phase cultures, they become heterogeneous. It is not the low pH of the sterile-filtrate that induces this change, since media buffered at the same pH as the sterile-filtrates are without effect (Table II). This fact suggests that substances produced in the medium of stationary phase cultures are capable of inducing the non-genetic physiological effect.

Table II. The Influence of pH and of the Sterile-Filtrate of a Heterogeneous Culture upon the Induction of Heterogeneity

Homogeneous Culture* Centrifuged and Resuspended in	Culture No.	No. at 0 hours			No. at 23 hours		
		G	C	G:C	G	C	G:C
Sterile-Filtrate of a Heterogeneous Culture, pH 5·5	A1	8·1	8·0	1·0	4·2	0·55	7·6
	A2	8·7	9·2	0·9	3·6	1·1	3·3
Synthetic Medium Minus Carbon Source, pH 6·8	B1	8·0	7·3	1·1	4·4	3·6	1·2
	B2	9·1	7·0	1·3	4·5	4·1	1·1
Synthetic Medium Minus Carbon Source, pH 5·5	C1	8·2	9·1	0·9	1·2	1·1	1·1
	C2	6·0	6·1	1·0	1·2	1·0	1·1

Values shown, except for ratios, are expressed in number of cells $\times 10^8$ per ml. Ratios greater than 1·5 are significant. Homogeneous cultures* were six similar cultures (unaerated 0·5% glucose) followed turbimetrically and centrifuged immediately upon attainment of final level of growth.

In order to analyze the curves represented in Figures 1 and 2, we follow the theory of ATWOOD and NORMAN[1] and assume that certain units are being inactivated in each cell and that (a) a cell will survive if at least one of its units survives and (b) the probability, S, that a unit survives a dose of radiation (or of the heterogeneity-inducing principle in stationary phase cultures), D, is given by the equation, $S = e^{-kD}$, where k is a constant. It can be shown that at sufficiently high dose the fraction of cells surviving, $S(n)$, is given by the equation, $S(n) = \bar{n} \cdot e^{-kD}$, where $\bar{n}$ is the average number of units per cell. Thus, by plotting log S versus D we should obtain, at high dose, a straight line which extrapolates back to give at $D = 0$ the average number of units per cell.

By referring to the Figures, it can be seen that the average number of units per cell, $\bar{n}$, whose inactivation, according to theory, results in the inability to grow in a medium containing a specific carbon source, turns out to be 1·7. A count of over 700 log phase cells, fixed and stained by the ROBINOW technique[2] to reveal the bacterial nuclei, gave an average number of nuclei per cell between 1·6 and 1·9, depending on the criteria used for distinguishing two nuclei from a "dividing" or enlarged single nucleus. The lower figure was obtained by counting as two only those nuclei which were distinctly separate; higher averages were obtained by using less stringent criteria. From these data and from the increasing evidence in molds[3] and yeasts[4] of a one-to-one correspondence between the number of units inactivated in cells by ultraviolet radiation and the number of nuclei per cell, it seems reasonable to conclude that the loss of ability of *Aerobacter aerogenes* cells to grow in media containing specific carbon sources is a consequence of the inactivation of their nuclei. This in turn would imply that non-heritable modifications can occur in nuclei or in intracellular units present in the same number as nuclei, such as genes in haploid cells.

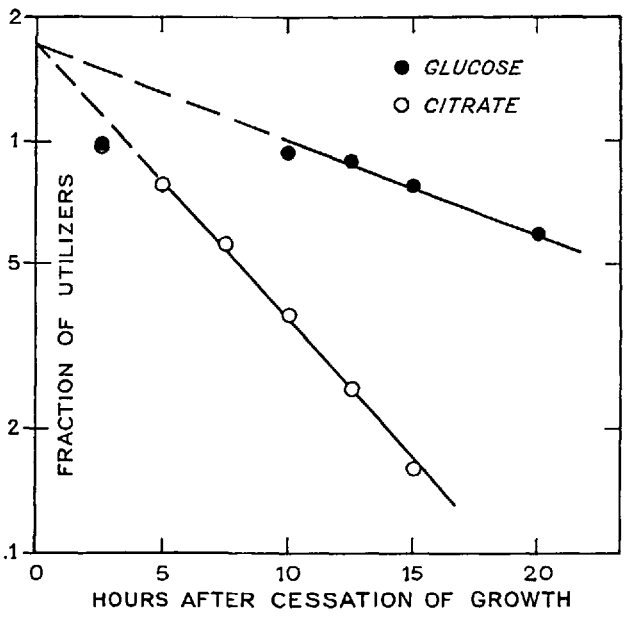

Fig. 1.—Decrease in glucose-utilizers and in citrate-utilizers in a population of *Aerobacter aerogenes* as a function of time after cessation of growth in an unaerated culture containing 0·5% glucose as sole carbon source. Growth is limited by pH.

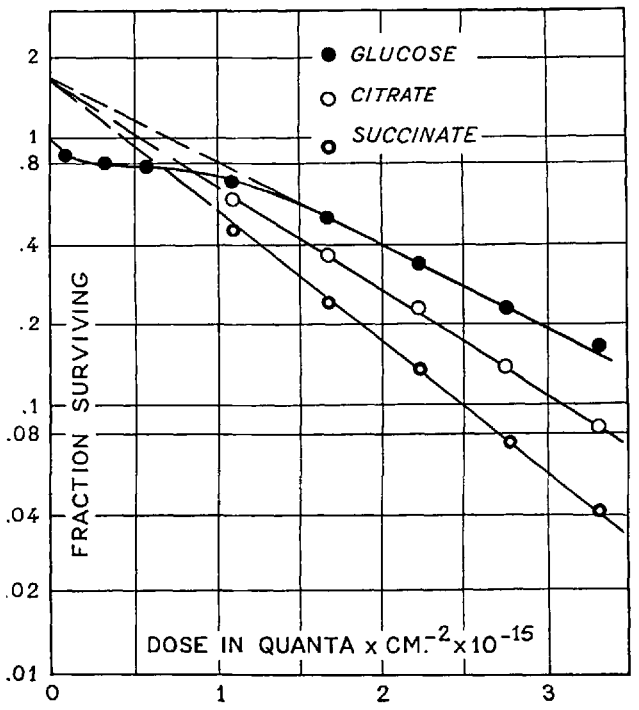

Fig. 2.—Inactivation of *Aerobacter aerogenes* by ultraviolet radiation. The inactivating wavelength is 254 mμ.

[1] K. C. ATWOOD and A. NORMAN, Proc. Nat. Acad. Sci. *35*, 696 (1949).

[2] C. F. ROBINOW, Addendum to *The Bacterial Cell* (Harvard University Press, 1947).

[3] A. NORMAN, Exp. Cell Research *2*, 454 (1951).

[4] R. LATARJET and B. EPHRUSSI, C. r. *229*, 306 (1949).—C.C. LINDEGREN, personal communication.

It remains to be seen (a) whether the inactivated cells have specifically lost the ability to metabolize the slow-adaptive substrates or (b) whether the inactivation of the nuclei can be differentially reversed by glucose, citrate, and succinate metabolism. Details of present and further investigations will be published elsewhere. Preliminary studies indicate that exposure to visible light can reverse the ultra-violet-induced physiological modification.

The work described in this note was performed in the Department of Zoology of Columbia University while one of us (A.W.R.) was a Fellow of the U.S. Public Health Service and the other (A.N.) was a Fellow of the Atomic Energy Commission.

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Résumé

Il est possible de provoquer, dans des populations d'*Aerobacter aerogenes*, à l'aide de conditions spéciales de culture, ou par irradiation ultra-violette, la perte de leur capacité de proliférer dans des milieux contenant des substrats carbonés adaptatifs. Cette perte n'est pas héréditaire. L'analyse cinétique du phénomène suggère que l'induction de cette modification est due à une inactivation facilement réversible du noyau des cellules.

Wachstumsversuche mit Myzelsuspensionen von *Mycelium Radicis atrovirens* in Ruhe- und Schüttelkulturen

Kürzlich haben WIKÉN und Mitarbeiter[1] eine einfache Methode entwickelt, welche zur Herstellung von Myzelsuspensionen für Wachstumsversuche mit Pilzen geeignet ist. Das Myzel wird dabei durch Schütteln mit Glaskugeln in destilliertem Wasser unter sterilen Bedingungen sehr fein verteilt. In Ruhekulturen, welche mit solchen Aufschwemmungen von Myzelfragmenten bzw. Hyphen geimpft werden, verläuft das Wachstum grundsätzlich in der gleichen Weise wie bei der Impfung nach der bisher üblichen Methode, das heisst mit Agarstückmyzel, das auf der Oberfläche der betreffenden Nährlösung schwimmend verbleibt. Wenn die Impfung mit angemessenen Mengen der Myzelsuspension erfolgt, kann bei zahlreichen Pilzen die gleiche Wachstumsgeschwindigkeit und die gleiche Höchstausbeute an Myzeltrockensubstanz festgestellt werden wie in den mit Agarstückmyzel geimpften Parallelkulturen. Die neue Impfungsmethode, deren Vorteile gegenüber der alten Agarstückmyzelmethode von WIKÉN und Mitarbeitern bereits diskutiert wurden, wird jetzt bei den im hiesigen Institute durchgeführten Untersuchungen über die physiologischen Eigenschaften mykorrhizabildender und streuzersetzender Pilze laufend angewendet (KELLER[2], SCHELLING[3], STÖCKLI[4]).

Verschiedene Forscher, zum Beispiel REITSMA[1] MODESS[2], LINDEBERG[3] und NORKRANS[4], haben hervorgehoben, dass das Myzel höherer Pilze in seiner Entwicklung von guter Sauerstoffzufuhr stark abhängig ist. MODESS[2], der das Wachstum des schwimmenden bzw. submersen Myzels von zwölf Pilzen untersuchte, konnte feststellen, dass nach Impfung mit dem ersten Myzel die Trockensubstanzproduktion bei den sechs Arten *Boletus bovinus, Boletus subtomentosus, Stropharia Hornemannii, Rhizopogon roseolus, Boletus luteus* und *Stropharia aeruginosa* 7- bis 22mal und bei den fünf Arten *Clitocybe clavipes, Clitocybe nebularis, Tricholoma albobrunneum, Tricholoma nudum* und *Lactarius deliciosus* 1,5–3,5mal grösser war als nach Impfung mit dem letzten Myzel. Nur bei einer Art, und zwar bei *Psalliota silvatica*, zeigte das submerse Myzel grössere Wachstumsgeschwindigkeit (2mal) als das schwimmende. Ohne Versuchswerte anzuführen, erwähnt LINDEBERG[3], dass bei gewissen der von ihm untersuchten Marasmiusarten schwimmende Myzelien um das mehrfache rascher wuchsen als submerse Myzelien. Wie bereits erwähnt, werden diese Unterschiede der Wachstumsgeschwindigkeit auf guten bzw. schlechten Luftzutritt zur Nährlösung der Kulturen sowie auf den ungleich grossen Sauerstoffbedarf der betreffenden Pilze zurückgeführt. REITSMA[1] und NORKRANS[4] geben weder Versuchswerte noch Schätzungen der Wachstumsgeschwindigkeit der untersuchten Pilze (*Armillaria mellea* bzw. Tricholomaarten) unter den verschiedenen Bedingungen an. In sämtlichen Untersuchungen kam als Impfmaterial Agarstückmyzel zur Verwendung.

Es sei ferner erwähnt, dass nicht nur Vertreter der Hymenomyzeten und Gasteromyzeten, sondern auch solche der niederen Pilze wegen des grossen Sauerstoffbedarfes des wachsenden Myzels bekannt sind. So zeigten beispielsweise bereits PASTEUR[5] und WEHMER[6], dass das Wachstum gewisser Mucorarten bei schlechtem Luftzutritt stark gehemmt wird, wobei das Myzel mehr oder weniger ausgeprägt das Aussehen einer «Kugelhefe» annimmt, indem durch Zerfall normaler, langgestreckter Hyphen kugelförmige Zellen entstehen, welche unter Umständen sogar zur Sprossung fähig sind. Der grosse Sauerstoffbedarf der bei den oxydativen Pilzgärungen wirksamen Arten der Gattungen *Aspergillus, Penicillium, Mucor, Rhizopus* usw. ist ein Faktor von grösster gärungstechnischer Bedeutung (vgl. BERNHAUER[7], PRESCOTT und DUNN[8], FOSTER[9]).

Mit Rücksicht auf die erwähnten Ansichten über den Sauerstoffbedarf verschiedener Vertreter der Bodenpilze schien es uns von Interesse zu sein, das Wachstum des submersen Myzels verschiedener Stämme von *Mycelium Radicis atrovirens* in Schüttelkulturen mit demjenigen in Ruhekulturen zu vergleichen. Das Studium der submersen Züchtung dieses Bodenpilzes in Schüttelkulturen war ferner von methodologischer Bedeutung für die im hiesigen Institute laufenden Untersuchungen über die Atmung der Pilze. Nach Impfung mit einer genügenden

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[3] G. LINDEBERG, Symb. Bot. Upsal. VIII:*2*, 41 (1944).
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