In this connection one should note that the factor of selection pressure will probably also vary where such isolation arises, as well as that of population size, for the populations will be held in somewhat different environments.

It may be noted in closing that here is a physiological factor acting on population mechanics that does not depend upon genetic change for changes in its specificity of action; in this respect it is similar to the homing reaction in salmon and birds, to the conditioned mating preferences of birds and to the reactions of ants toward colony mates.

4 Taft, A. C., and Shapovalov, L., Calif. Fish and Game, 24, 118-125 (1938).
5 Cushing, J. E., Condor, 43, 103-107 (1941).
6 Cushing, J. E., Ibid., 43, 233-236 (1941).
specificities are of the same order. There are, however, a number of limitations inherent in this approach. Perhaps the most serious of these is that the investigator must in general confine himself to a study of non-lethal heritable characters. Such characters are likely to involve more or less non-essential so-called "terminal" reactions. The selection of these for genetic study was perhaps responsible for the now rapidly disappearing belief that genes are concerned only with the control of "superficial" characters.

Considerations such as those just outlined have led us to investigate the general problem of the genetic control of developmental and metabolic reactions by reversing the ordinary procedure and, instead of attempting to work out the chemical bases of known genetic characters, to set out to determine if and how genes control known biochemical reactions. The ascomycete Neurospora offers many advantages for such an approach and is well suited to genetic studies. Accordingly, our program has been built around this organism. The procedure is based on the assumption that x-ray treatment will induce mutations in genes concerned with the control of known specific chemical reactions. If the organism must be able to carry out a certain chemical reaction to survive on a given medium, a mutant unable to do this will obviously be lethal on this medium. Such a mutant can be maintained and studied, however, if it will grow on a medium to which has been added the essential product of the genetically blocked reaction. The experimental procedure based on this reasoning

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**TABLE 1**

Growth of Pyridoxinless Strain of *N. sitophila* on Liquid Medium Containing Inorganic Salts, 1% Sucrose, and 0.004 Microgram Biotin per Cc. Temperature 25°C. Growth Period, 6 Days from Inoculation with Conidia

<table>
<thead>
<tr>
<th>Micrograms Bi per 25 cc. medium</th>
<th>Strain</th>
<th>Dry weight mycelia, mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>76.7</td>
</tr>
<tr>
<td>0</td>
<td>Pyridoxinless</td>
<td>1.0</td>
</tr>
<tr>
<td>0.01</td>
<td></td>
<td>4.2</td>
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<tr>
<td>0.03</td>
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<td>25.5</td>
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<td>65.4</td>
</tr>
<tr>
<td>30.0</td>
<td></td>
<td>82.4</td>
</tr>
</tbody>
</table>
can best be illustrated by considering a hypothetical example. Normal strains of *Neurospora crassa* are able to use sucrose as a carbon source, and are therefore able to carry out the specific and enzymatically controlled

![Graph](image_url)

**FIGURE 1**

Growth of normal (top two curves) and pyridoxinless (remaining curves) strains of *Neurospora sitophila* in horizontal tubes. The scale on the ordinate is shifted a fixed amount for each successive curve in the series. The figures at the right of each curve indicate concentration of pyridoxine (B₆) in micrograms per 25 cc. medium.

reaction involved in the hydrolysis of this sugar. Assuming this reaction to be genetically controlled, it should be possible to induce a gene to mutate to a condition such that the organism could no longer carry out sucrose hydrolysis. A strain carrying this mutant would then be unable to grow
on a medium containing sucrose as a sole carbon source but should be able
to grow on a medium containing some other normally utilizable carbon
source. In other words, it should be possible to establish and maintain
such a mutant strain on a medium containing glucose and detect its
inability to utilize sucrose by transferring it to a sucrose medium.

Essentially similar procedures can be developed for a great many meta-
'bolic processes. For example, ability to synthesize growth factors (vi-
tamins), amino acids and other essential substances should be lost through
gene mutation if our assumptions are correct. Theoretically, any such
metabolic deficiency can be "by-passed" if the substance lacking can be
supplied in the medium and can pass cell walls and protoplasmic mem-
branes.

In terms of specific experimental practice, we have devised a procedure
in which x-rayed single-spore cultures are established on a so-called "com-
plete" medium, i.e., one containing as many of the normally synthesized
constituents of the organism as is practicable. Subsequently these are
tested by transferring them to a "minimal" medium, i.e., one requiring the
organism to carry on all the essential syntheses of which it is capable.
In practice the complete medium is made up of agar, inorganic salts, malt
extract, yeast extract and glucose. The minimal medium contains agar
(optional), inorganic salts and biotin, and a disaccharide, fat or more
complex carbon source. Biotin, the one growth factor that wild type
*Neurospora* strains cannot synthesize, is supplied in the form of a com-
mercial concentrate containing 100 micrograms of biotin per cc. Any
loss of ability to synthesize an essential substance present in the complete
medium and absent in the minimal medium is indicated by a strain growing
on the first and failing to grow on the second medium. Such strains are
then tested in a systematic manner to determine what substance or sub-
stances they are unable to synthesize. These subsequent tests include
attempts to grow mutant strains on the minimal medium with (1) known
vitamins added, (2) amino acids added or (3) glucose substituted for the
more complex carbon source of the minimal medium.

Single ascospore strains are individually derived from perithecia of *N.
crassa* and *N. sitophila* x-rayed prior to meiosis. Among approximately
2000 such strains, three mutants have been found that grow essentially
normally on the complete medium and scarcely at all on the minimal
medium with sucrose as the carbon source. One of these strains (*N.
sitophila*) proved to be unable to synthesize vitamin B₈ (pyridoxine). A
second strain (*N. sitophila*) turned out to be unable to synthesize vitamin
B₁ (thiamine). Additional tests show that this strain is able to synthesize
the pyrimidine half of the B₁ molecule but not the thiazole half. If
thiazole alone is added to the minimal medium, the strain grows essen-
tially normally. A third strain (*N. crassa*) has been found to be unable
to synthesize para-aminobenzoic acid. This mutant strain appears to be entirely normal when grown on the minimal medium to which \( p \)-aminobenzoic acid has been added. Only in the case of the "pyridoxinless" strain has an analysis of the inheritance of the induced metabolic defect been investigated. For this reason detailed accounts of the thiamine-deficient and \( p \)-aminobenzoic acid-deficient strains will be deferred.

Qualitative studies indicate clearly that the pyridoxinless mutant, grown on a medium containing one microgram or more of synthetic vitamin \( B_6 \) hydrochloride per 25 cc. of medium, closely approaches in rate and characteristics of growth normal strains grown on a similar medium with

![Graph showing the relation between growth rate (cm./day) and vitamin \( B_6 \) concentration.]

no \( B_6 \). Lower concentrations of \( B_6 \) give intermediate growth rates. A preliminary investigation of the quantitative dependence of growth of the mutant on vitamin \( B_6 \) in the medium gave the results summarized in table 1. Additional experiments have given results essentially similar but in only approximate quantitative agreement with those of table 1. It is clear that additional study of the details of culture conditions is necessary before rate of weight increase of this mutant can be used as an accurate assay for vitamin \( B_6 \).

It has been found that the progression of the frontier of mycelia of *Neurospora* along a horizontal glass culture tube half filled with an agar medium provides a convenient method of investigating the quantitative
effects of growth factors. Tubes of about 13 mm. inside diameter and about 40 cm. in length are used. Segments of about 5 cm. at the two ends are turned up at an angle of about 45°. Agar medium is poured in so as to fill the tube about half full and is allowed to set with the main segment of the tube in a horizontal position. The turned up ends of the tube are stoppered with cotton plugs. Inoculations are made at one end of the agar surface and the position of the advancing front recorded at convenient intervals. The frontier formed by the advancing mycelia is remarkably well defined, and there is no difficulty in determining its position to within a millimeter or less. Progression along such tubes is strictly linear with time and the rate is independent of tube length (up to 1.5 meters). The rate is not changed by reducing the inside tube diameter to 9 mm., or by sealing one or both ends. It therefore appears that gas diffusion is in no way limiting in such tubes.

The results of growing the pyridoxinless strain in horizontal tubes in which the agar medium contained varying amounts of B₆ are shown graphically in figures 1 and 2. Rate of progression is clearly a function of vitamin B₆ concentration in the medium.¹⁰ It is likewise evident that there is no significant difference in rate between the mutant supplied with B₆ and the normal strain growing on a medium without this vitamin. These results are consistent with the assumption that the primary physiological difference between pyridoxinless and normal strains is the inability of the former to carry out the synthesis of vitamin B₆. There is certainly more than one step in this synthesis and accordingly the gene differential involved is presumably concerned with only one specific step in the biosynthesis of vitamin B₆.

<table>
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<th>4</th>
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<tbody>
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<td>17</td>
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<td>N</td>
<td></td>
<td>pdx</td>
<td>pdx</td>
<td>pdx</td>
</tr>
</tbody>
</table>

N, normal growth on B₆-free medium. pdx, slight growth on B₆-free medium. Failure of ascospore germination indicated by dash.

* Spores 2, 3 and 4 isolated but positions confused. Of these, two germinated and both proved to be mutants.
In order to ascertain the inheritance of the pyridoxinless character, crosses between normal and mutant strains were made. The techniques for hybridization and ascospore isolation have been worked out and described by Dodge, and by Lindegren. The ascospores from 24 asci of the cross were isolated and their positions in the asci recorded. For some unknown reason, most of these failed to germinate. From seven asci, however, one or more spores germinated. These were grown on a medium containing glucose, malt-extract and yeast extract, and in this they all grew normally. The normal and mutant cultures were differentiated by growing them on a B₆ deficient medium. On this medium the mutant cultures grew very little, while the non-mutant ones grew normally. The results are summarized in table 2. It is clear from these rather limited data that this inability to synthesize vitamins B₆ is transmitted as it should be if it were differentiated from normal by a single gene.

The preliminary results summarized above appear to us to indicate that the approach outlined may offer considerable promise as a method of learning more about how genes regulate development and function. For example, it should be possible, by finding a number of mutants unable to carry out a particular step in a given synthesis, to determine whether only one gene is ordinarily concerned with the immediate regulation of a given specific chemical reaction.

It is evident, from the standpoints of biochemistry and physiology, that the method outlined is of value as a technique for discovering additional substances of physiological significance. Since the complete medium used can be made up with yeast extract or with an extract of normal Neurospora, it is evident that if, through mutation, there is lost the ability to synthesize an essential substance, a test strain is thereby made available for use in isolating the substance. It may, of course, be a substance not previously known to be essential for the growth of any organism. Thus we may expect to discover new vitamins, and in the same way, it should be possible to discover additional essential amino acids if such exist. We have, in fact, found a mutant strain that is able to grow on a medium containing Difco yeast extract but unable to grow on any of the synthetic media we have so far tested. Evidently some growth factor present in yeast and as yet unknown to us is essential for Neurospora.

Summary.—A procedure is outlined by which, using Neurospora, one can discover and maintain x-ray induced mutant strains which are characterized by their inability to carry out specific biochemical processes.

Following this method, three mutant strains have been established. In one of these the ability to synthesize vitamin B₆ has been wholly or largely lost. In a second the ability to synthesize the thiazole half of the vitamin B₁ molecule is absent, and in the third para-aminobenzoic acid is not
synthesized. It is therefore clear that all of these substances are essential growth factors for \textit{Neurospora}.\footnote{11}

Growth of the pyridoxinless mutant (a mutant unable to synthesize vitamin B$_6$) is a function of the B$_6$ content of the medium on which it is grown. A method is described for measuring the growth by following linear progression of the mycelia along a horizontal tube half filled with an agar medium.

Inability to synthesize vitamin B$_6$ is apparently differentiated by a single gene from the ability of the organism to elaborate this essential growth substance.

**Note:** Since the manuscript of this paper was sent to press it has been established that inability to synthesize both thiazole and $p$-aminobenzoic acid are also inherited as though differentiated from normal by single genes.

* Work supported in part by a grant from the Rockefeller Foundation. The authors are indebted to Doctors B. O. Dodge, C. C. Lindegren and W. S. Malloch for stocks and for advice on techniques, and to Miss Caryl Parker for technical assistance.

\footnote{1} The possibility that genes may act through the mediation of enzymes has been suggested by several authors. See Troland, L. T., \textit{Amer. Nat.}, 51, 321–350 (1917); Wright, S., \textit{Genetics}, 12, 530–569 (1927); and Haldane, J. B. S., in \textit{Perspectives in Biochemistry}, Cambridge Univ. Press, pp. 1–10 (1937), for discussions and references.

\footnote{2} Ouslow, Scott-Moncrieff and others, see review by Lawrence, W. J. C., and Price, J. R., \textit{Biol. Rev.}, 15, 35–58 (1940).


\footnote{7} In so far as we have carried them, our investigations on the vitamin requirements of \textit{Neurospora} corroborate those of Butler, E. T., Robbins, W. J., and Dodge, B. O., \textit{Science}, 94, 262–263 (1941).

\footnote{8} The biotin concentrate used was obtained from the S. M. A. Corporation, Chagrin Falls, Ohio.

\footnote{9} Throughout our work with \textit{Neurospora}, we have used as a salt mixture the one designated number 3 by Fries, N., \textit{Symboles Bot. Upsalienses}, Vol. 3, No. 2, 1–188 (1938). This has the following composition: NH$_4$ tartrate, 5 g.; NH$_2$NO$_3$, 1 g.; KH$_2$PO$_4$, 1 g.; MgSO$_4$·7H$_2$O, 0.5 g.; NaCl, 0.1 g.; CaCl$_2$, 0.1 g.; FeCl$_3$, 10 drops 1% solution; H$_2$O, 1 l. The tartrate cannot be used as a carbon source by \textit{Neurospora}.

\footnote{10} It is planned to investigate further the possibility of using the growth of \textit{Neurospora} strains in the described tubes as a basis of vitamin assay, but it should be emphasized that such additional investigation is essential in order to determine the reproducibility and reliability of the method.

\footnote{11} The inference that the three vitamins mentioned are essential for the growth of normal strains is supported by the fact that an extract of the normal strain will serve as a source of vitamin for each of the mutant strains.