March 4, 1950

To the Committee on Graduate Study:

I am submitting to you a thesis written by Frank M. Boyd entitled "Induced Mutations In Molds." I recommend that it be accepted for nine quarter hours credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Bacteriology.

Arthur L. Pollard
Major Professor

We have read this thesis
and recommend its acceptance:

Herman C. Lichstein

William B. Cherry

Accepted for the Committee

E. A. Walters
Dean of the Graduate School

INDUCED MUTATIONS IN MOLDS

A THESIS

Submitted to
The Committee on Graduate Study
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Master of Science

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by

Frank M. Boyd

March 1950

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F.M.B.

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INTRODUCTION

It has long been known that microorganisms will dissociate into forms which do not resemble the parent strain in one or more properties. Hadley (1927) defines dissociation as involving the partial or total transformation of a pure strain of a normal type into one or more subtypes often differing in cultural, morphological, serological, and biochemical characters from the original. The phenomenon may be accompanied by the disappearance of the parent strain. If this transformation takes place rapidly, the parent strain may lyse and disappear.

On solid medium the following may occur:

1. Secondary colony formation without erosive action.
2. Secondary colony formation with erosive action.
3. Lysis and transformation over limited or broad areas without erosive features and with delayed secondary colony formation.

When secondary colonies form, the old colony usually becomes translucent over the entire surface, and papillae arise over the surface.

The first important work on this subject was done by Neisser in 1906, according to Hadley (1927) and Lewis (1933), when he described what is now known as Bacterium coli-mutabile, which does not ferment lactose but gives rise to a subrace which does. This occurs in broth and on lactose agar by forming papillae. This is perhaps the best example of secondary colony

formation, and is typical of this phenomenon.

In this laboratory, it was noticed that stock cultures of Escherichia coli, after being stored in the refrigerator for a number of weeks and then replaced in the incubator, developed these typical papillae on the surface of the old growth. It was believed that perhaps this type of growth might be analagous to cancerous growth in higher type tissue, since physical irritation or stimulation is believed to be one of the causes of cancer. In order to test this theory, it was decided to use some known carcinogenic drugs on the organisms and ascertain if variants or secondary colonies would be formed.

Published work on these carcinogenic drugs yields somewhat contradictory results. Numerous studies have shown that addition of certain of the substances to the medium will stimulate the growth of bacteria and yeasts. Goldstein (1937) reports 50 percent more organisms in the eight to nine hour growth of E. coli in the presence of 1,2,5,6-dibenzanthracene. Hopper and Clapp (1939) found similar results using methylcholanthrene and 1,2,5,6-dibenzanthracene, but got no stimulation using certain other drugs, including phenanthrene and 1,2 benzanthracene. Cook, Hart and Joly (1938) report stimulation of yeast growth with 1,2,5,6-dibenzanthracene at a concentration of 9×10^{-4} molar. Other concentrations had less effect. However, Dodge, Dodge, and Johnson (1941) found that only a slight difference exists between methylcholanthrene-treated and control cultures, as far as growth rate, numbers, or mutations were con-

cerned, using Saccharomyces ellipsoideus as test organism. Fishbein, Weaver, and Scherago (1941), using a colloidal suspension of 1,2,5,6-dibenzanthracene and E. coli, found that using large inocula caused the maximal count to be much higher, but found no increase in production of variants. Latarjet (1948) used a number of carcinogenic and non-carcinogenic substances on E. coli and found that there is no apparent relation between carcinogenic and mutagenic activities in the production of a phage-resistant form of that organism.

Thom and Steinberg (1939) investigated the mutagenic properties of a number of substances with the hope of contributing toward a more complete taxonomic knowledge of certain Aspergilli, and to develop more efficient industrial strains. They used many different drugs and dyes in their culture solutions, including colchicine and 85 phenanthrene derivatives, of which the carcinogens were members. They found that growing A. niger on agar slants containing aniline blue, acridine yellow, methylene blue and methylene violet gave vigorously-growing strains with yellow mycelia and retarded sporulation. These results could not be duplicated at will, indicating the presence of some unknown factors.

In a later article, Steinberg and Thom (1940) report that mannitol and sodium nitrite produces "injury" mutants in Aspergilli. They believe this action is due to the nitrous acid that is formed stripping amino groups from genetically important amino acids or proteins in the mold cells. They

also found that colchicine, methylcholantrene, and 1,2,5,6-dibenzanthracene, produced variants.

The mechanism by which the carcinogenic and other drugs stimulate the rate of growth of microorganisms and induce variation is at the present quite obscure. It is not known what type of chemical structure, if any, is stimulatory toward mutation production, though it is known that the 1,2 benzanthracene ring system with a carbon substituent at the meso position 10 is the most significant portion of the carcinogens as far as cancer-producing ability goes. Other compounds with similar structures have carcinogenic action. But, as has been stated, there is no apparent relation between carcinogenic and mutagenic properties of these drugs.

A source of difficulty in using these hydrocarbons in microbiological work is their insolubility in water, and thus in culture media. In using these drugs superficially on animals, a suspension in oil serves the purpose satisfactorily, but in bacteriological cultures, the drugs are not soluble, rendering quantitative measurement of treatment of cultures inaccurate and difficult.

It was decided to test the mutagenic effect of ultraviolet light, one of the most common and simplest methods of obtaining mutations in bacteria and molds. It has long been known that ultraviolet radiations would kill microorganisms with intensive treatment, and induce mutations with less treatment.

Workers seem to agree that the wave length of the radiation is quite important in determining the sterilizing or mutagenic efficiency of the treatment. Hollaender and Emmons, (1945), and Hollaender, et. al. (1945) determined that the most effective range for killing and mutation production is 2000 to 3000 Angstroms. The most effective wave length is 2650 Angstroms. They state that at 2650 Angstroms the nucleic acids have their highest absorption. At this point the effects of the irradiation reached the maximum. At 2280 Angstrom units, the nucleic acids, proteins and other constituents absorb radiation intensely, but the effect on the organisms is killing rather than mutation. At 2967 Angstroms there is very little absorption, but high doses can cause killing or mutation. These authors state that X-rays leave more survivors and create more mutants, especially sterile mutants, than ultraviolet.

Of principal interest in this study are morphological variants, which is the most common type described by most workers. In general, the mutants, or as they are called by some, saltants, show variation in color or amount of pigments and increased or decreased production of aerial hyphae and spores. Growth and colony size is often affected by radiations. Kelner (1948) obtained three mutants by treating Streptomyces flaveolus with X-rays and ultraviolet light. He described them as being "yellow," "asporogeneous," and "restricted," the latter growing more poorly on asparagine glucose agar

than the parent strain. Kelner states that X-rays gave more uniform results than ultraviolet and it is the preferred agent for inducing mutation in Actinomycetes.

Whelden (1940) irradiated A. niger spores with low voltage cathode rays. The variants he obtained differed from the parent strain most commonly in the color of the fruiting mycelium and by increased size. At the voltage used, the energy was released mostly in the zone of the nucleus, so he concluded that these nuclear changes were mutations. A cytological examination showed only one evident nuclear change: in the large variants the number of chromosomes is twice that of the normal form. He described five types of variants, one of which occured only twice. Early in its development the mycelium showed a brilliant "citron yellow" color. As the culture matured, its older portions became "pyrite yellow" in color. The mature spores were brownish in color and much darker than the mycelium. This description is quite similar to a variant obtained in this study, and to these described above by Thom and Steinberg.

Raper, Coghill and Hollaender (1945) also found deficiency mutations in A. terreus such as are so often found in Neurospora. In one case the organism required ammonia nitrogen for typical growth.

Little work has been reported toward identifying the pigments found in various fungi. Most attention has been paid to merely noting the presence or absence of a particular color in species. There is a wide variety of pigmentation in fungi,

the A. niger group being no exception. However, in this group, Thom and Raper (1945) state that the pigment seems to be a strain or race character little influenced by handling which is not destructive to the race entity. Each race, according to them, seems to reach a fixed quantitative limit in the secretion of the coloring substance, thus reducing the most conspicious diagnostic character among closely related forms to a quantitative rather than a qualitative basis of separation.

Thom and Raper noted that most Aspergillus species, if they produce color in the substratum, produce from a trace to abundant yellow in the early stages. This may persist or give way to shades of orange, red or purple, or fade away. No intensive work has apparently been done on any pigment of this series except aspergilline.

Perhaps if more study were given to pigment production, and to detailed morphological and physiological study of mutations, more useful taxonomic information might be obtained and applied to a group where there is great need of establishing relationship and differentiation between species, as in the A. niger group. It is hoped that this and other work will contribute a little of that information, or serve to direct further investigation which will yield more knowledge toward that end.

EXPERIMENTAL PROCEDURE AND RESULTS

The following carcinogenic drugs were used: methylcholanthrene, 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, p-aminodimethylaniline oxalate, and dimethylaminoazobenzene (butter yellow). All were products of the Eastman Kodak Company, Rochester, N. Y. The bacteria used were *Escherichia coli*, strains 10B3, H52, and Tennessee; and *Aerobacter aerogenes* strains W and D2. All were obtained from the departmental stock cultures.

The problem of dissolving the materials in the culture medium was encountered from the first. The hydrocarbons were soluble only in such materials as benzene or hot alcohol. The butter yellow proved to be soluble in oil, alcohol, benzene, and chloroform. None were water soluble, and therefore insoluble in the medium.

The method employed was to sterilize 45 milligrams of the chemical in a test tube and add it to 150 milliliters of nutrient broth, giving a concentration of 0.3 percent. The broth was then inoculated with the organism and placed on a shaker, which served to keep the drug reasonably well suspended in the solution. After 24 and 48 hours incubation, samples would be removed, diluted and placed on nutrient and litmus lactose agar. The plates would then be incubated and examined for morphological and biochemical variants. The latter were examined for abnormal lactose fermentation only.

In addition, plates of nutrient agar containing the same concentration of the drugs were inoculated with the organisms, but again the drugs were merely suspended in the medium and did not go into solution. Neither method yielded any apparent variants after many trials.

It was then decided to test the same materials using the department strain of Aspergillus niger. All transfers were made to Czapek solution agar, which has the following composition:

	Percent
Sodium nitrate	0.2
Potassium dihydrogen phosphate	0.1
Potassium chloride	0.05
$MgSO_4 \cdot 7H_2O$	0.05
Ferrous Sulphate	0.001
Sucrose	3.0
Agar	1.5
Distilled water	1000 Ml.

The plates were inoculated with spores of A. niger, either by streaking or pouring, and were incubated for 2 to 3 days at room temperature. Then several sterile filter paper discs such as are used in antibiotic assays were placed on the surface of the agar. Onto these discs was dropped a solution of the substance being tested. The plates were then left at room temperature until the mold had reached maturity and sporulated, usually 2 or 3 days later. The plates were examined under the microscope daily until cessation of growth. The examination included both

colony color and shape and the shape of the individual conidial heads.

The drugs used were the same as those listed before plus a 0.3 percent solution of colchieine. Solvents used included alcohol, benzene, and olive oil. No attempt was made here to accurately measure the concentration, as the drug was absorbed by the disc as the solvent evaporated. The hydrocarbons were insoluble in the olive oil, but the treatment was used since vegetable oils reportedly augment carcinogenesis while oils from animal sources retard it.

One of the greatest disadvantages encountered in this study was the great natural variation in the shapes of the conidial heads in the A. niger. They ranged from small, globose heads to large heads shaped like a shock of wheat, called divergent columns. On a typical plate, several types of conidial heads could be found, thus making it impossible to differentiate natural variants from induced ones. No variation in conidial formation, in pigmentation, or in any other characteristic was apparent after a quite intensive study.

Ultraviolet light was used as a means of qualitatively determining some of the morphological mutants which might be obtained with such treatment. The radiations were obtained from a General Electric lamp, type T-5, rated at eight watts at three inches. It produced 860 ergs/sec./cm.^{2}. The effective radiation was in the range of 2650 Angstrom units.

The method of treating the spores was as follows: a

Czapek solution agar slant of a 2 to 3 day old culture was covered with 3 ml. of sterile distilled water and the spores removed by gentle scraping with a wire loop. The resulting suspension was then placed in a sterile 25 ml. quartz flask and stoppered. No attempt was made to count or standardize the numbers of spores in the suspension. The flask was placed horizontally on a board with an electric motor which turned the flask at a high rate of speed, keeping a uniform suspension against the center of the inner surface of the flask and thus giving each spore an equal exposure to the radiation. The lamp was placed three inches from the top of the flask. After the desired exposure, the suspension was diluted and plated on Czapek solution agar. After the colonies had developed, in about two days, they were then examined daily throughout their development for the appearance of visible mutations.

Spores were irradiated a total of 19 times, with 6 to 8 plates being made of each suspension to give representative samples. Only three colonies were found which exhibited obvious morphological differences from the parent organism. One of these was found on a plate from a suspension of spores from a two-day-old culture of A. niger which was exposed to ultra-violet for 21 minutes. The colony was hard, with white mycelia. On subsequent transfers, the variant produced white mycelia with black conidial heads. Conidial formation was somewhat restricted with the heads being somewhat larger than normal. Growth of the mutant strain was not as rapid as that of the normal strain.

The other two variants were identical in appearance. Each had yellow mycelia with normal conidia formation. One was obtained from a 3-day-old suspension irradiated for 25 minutes, the other from a 2-day-old culture exposed for 28 minutes. Growth was comparable to the normal strain. The mutants could be propagated by the transfer of spores or mycelia. The cultures are still in stock, although they frequently and unpredictably revert to the normal type colony with grayish mycelia. In addition, the white mutant on transfer sometimes grows out as the yellow form.

Cultures of the variant strains and controls were made in Czapek's solution containing several carbohydrate sources. Growth of the variants was less than that of the control in 48 hours. Carbohydrates used were: levulose, glucose, arabinose, starch, galactose, maltose, xylose, mannitol, glycerol, and sucrose.

It was found that when the yellow organisms were cultivated in Czapek solution in shake cultures, the pH of the media was lowered to considerably less than the control cultures, as is shown in Table I. The pH was measured on the Beckman pH meter.

Inasmuch as no published work has been done on the identification of the yellow pigment of this mutation of A. niger, it was decided to attempt to isolate it and determine its chemical nature as far as possible. It was noticed at once that there were apparently two types of yellow pigments, one type being

TABLE I

pH OF MEDIA AFTER 48 HOURS GROWTH

	Experiment Number		
	1	2	3
Before Growth	3.5	4.6	3.9
Mutant Strain	1.7	1.7	1.4
Parent Strain	2.6	2.6	3.4

soluble in the media and the other insoluble. Work was confined to the isolation and identification of the water insoluble pigment.

The extraction procedure employed is shown in Figure 1. The procedure was determined by testing the solubility of the pigmented substances at each step in various types of solvents. The pigment was first extracted with hot methanol by macerating the mold in a mortar and placing the material on a filter. The alcohol was poured over the mold until most of the color was removed. The alcohol was evaporated under vacuum with heat from a heat lamp to prevent decomposition of organic materials. From solubility tests, it was believed that the pigment was of fatty acid structure. Therefore, a saturated solution of potassium hydroxide in methanol was used to accomplish the saponification. Time of treatment varied from one hour to overnight with little visible effect on results. When the soaps were purified and then acidified with hydrochloric acid, some bright yellow material rose to the top of the solution, thus indicating that the pigment was of fatty acid composition. There was considerable material which was unsaponifiable. Some of this material was soluble in chloroform and gave a positive test for the presence of sterols.

In a further effort to determine the type of steroids present, several group-specific tests were used. One was the Liebermann-Burchard reaction, which consists of treating the sterol in chloroform with acetic anhydride and sulphuric acid.

A heterochromatic reaction results with cholesterol, but not with cholestanol or coprostanol. The sterol portion of mold extract gave a dark red color, indicative of the presence of cholesterol-like material. The Rosenheim reaction indicates the presence of a diene system (ergosterol) or those systems capable of forming such a system by dehydration with trichloroacetic acid (-cholestanol). The reaction consists of adding a few drops of trichloroacetic acid to a chloroform solution of the sterol. The mold extract gave a negative reaction in this test.

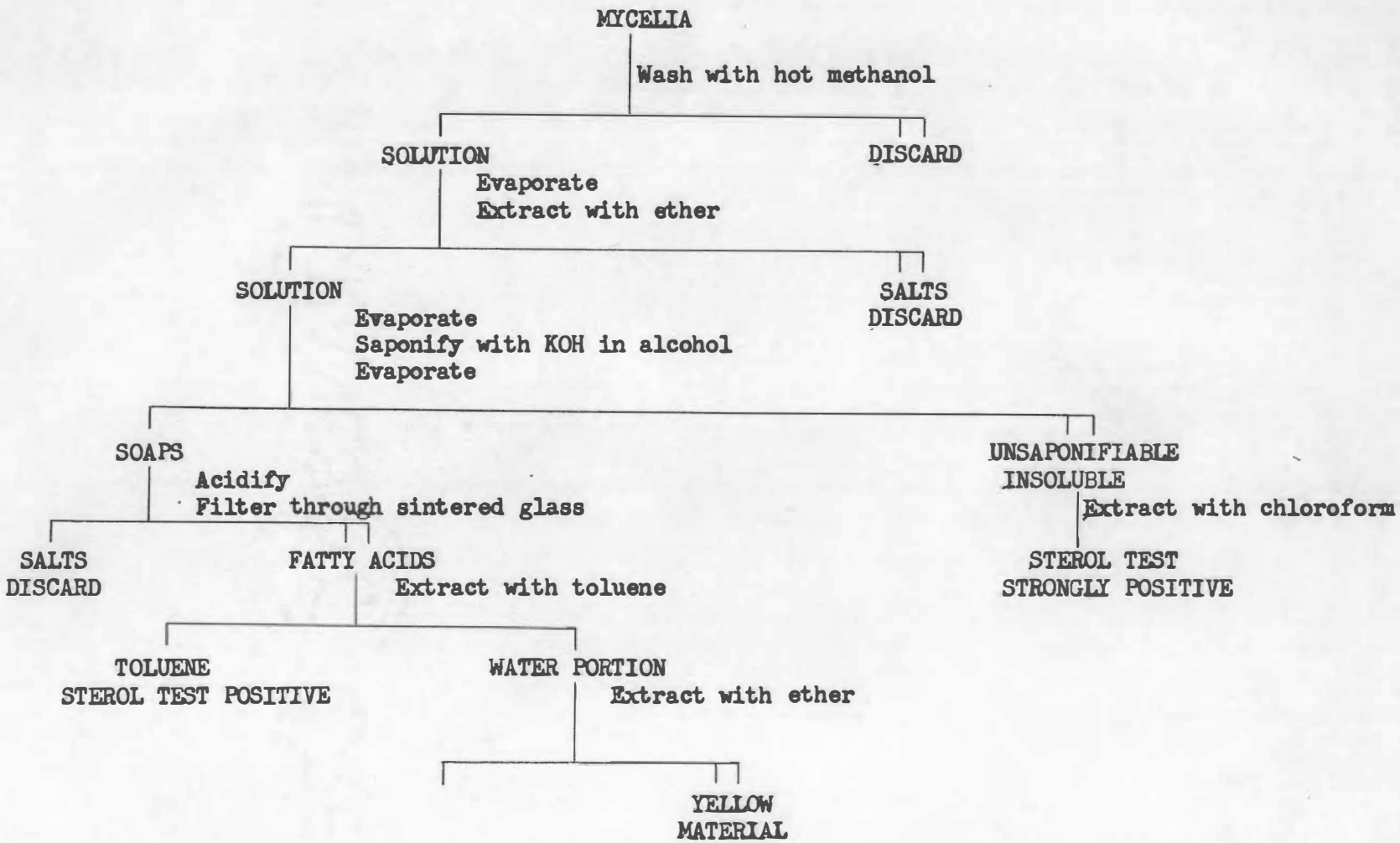

Figure 1. Separation of pigment from the mycelia of A. Niger

DISCUSSION

An examination of the results obtained with the carcinogenic drugs indicates that these drugs are ineffectual in stimulating mutation production under the experimental conditions used. As stated previously, they are insoluble in culture medium and thus microorganisms do not interact with them to a great extent. The literature provides evidence of the growth-stimulating properties of some of these materials, but there is little evidence that carcinogenic materials are mutagenic due to their carcinogenic properties. If this were true, some correlations might be drawn concerning carcinogenesis in animal tissue and mutation in molds and bacterial cells. But apparently, as shown by this and other work, the mechanisms of carcinogenesis and mutation are entirely different. It is conceivable that other methods of treating microorganisms with these chemicals may yield entirely different results from these.

The colchicine used for treatment of the *A. niger* spores was water-soluble and, if mutagenic, should have caused formation of variants in this experiment. However, the concentration was very dilute and relatively few spores were exposed to it, as compared to the number of spores and quantity of treatment using ultraviolet. If the ratio of mutations from colchicine or any of the other drugs were no higher than that obtained from ultraviolet irradiation, then it would take many plates and discs to demonstrate even one variant. In plating

spores exposed to ultraviolet, all the spores have been equally treated and each is a potential source of a mutation, but with the disc method many organisms have not been touched by the chemical and the concentration of the chemical varies with the distance from the disc. Also, in the disc technique employed here, the drug was not applied until the organisms had already started growing. Thus, a variation in one cell of a mold could be masked by the rest of the normal cells.

The ultraviolet produced typical variants, though the number did not approach the 40 percent mutation rate described by Hollaender and Emmons (1946). Since only 3 variant colonies were found in well over a hundred plates. Nevertheless, the evidence indicates that ultraviolet irradiation is the most efficient of several methods studied of the production of mutants. Its effectiveness is possibly exceeded only by X-rays.

One problem which has not been solved is the reversion of the white strain to the yellow form and the change of both back to the normal form. This occurred frequently and it was with difficulty that strains were kept pure in broth cultures on many occasions. This is probably due to recombination or some other genetic activity causing loss of the variant properties. This is a problem which all workers with mutations must face and it often causes difficulty in propagating mutants.

The yellow mutation shows higher production of pigment as compared with the parent organism, rather than gaining the ability to produce a completely new product. The parent or-

ganism often shows a small amount of yellow color in the reverse mycelia, though the amount does not approach that found in the mutation. The pigment is apparently of fat or fatty acid composition, as shown by the separation and solubility tests. This technique further illustrates the possible industrial importance of ultraviolet irradiation of mold spores to create stable mutants which produce an increased amount of some desirable product. The separation technique employed here appears to be effective for separating the sterols from the other materials, as well as removing the pigment.

The fact that the mutant lowered the pH of the medium considerably below that of the original strain is of interest in addition to the increased pigmentation. This indicates some biochemical variation which may be of industrial or academic interest.

The biochemical studies of these mutants are by no means complete. The pigments and steroids have been studied qualitatively but have not been specifically identified. One of the variations has not been studied at all. It does not appear to have the acid-producing properties of the yellow strain, however, and is not as stable on transfer.

It is hoped that the description of these mutants of *A. niger*, techniques for extraction of pigments and use of certain chemicals has yielded information of value in the study of mutagenesis.

SUMMARY

This experiment was originally intended to investigate the mutagenic effect of certain carcinogenic drugs, and to determine the possible relationship between mutation in microorganisms and cancer in higher tissue. Attempts to produce visible morphological mutations or modified lactose fermentation in *Escherichia coli* and *Aerobacter aerogenes* by placing the various drugs in the culture media did not yield any mutations. These same materials were then used on *Aspergillus niger*, by placing the chemical on absorbent discs on petri plates and examining the mold in the surrounding area for variations. This procedure also failed to yield mutations.

Spores of *Aspergillus niger* were irradiated with ultraviolet light and plated. The resulting colonies were examined for morphological mutations. Three such variations were found and described. The processes for extraction of the pigment of one of the variants and the chemical identification of it and other components of the mycelia are described.

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