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28 May 1947

I hereby recommend that the thesis prepared under my supervision by Violet Marion Tiller

entitled Studies of a Soft X-Ray Mutant of Aspergillus niger van Tieghem

be accepted as fulfilling this part of the requirements for the degree of Ph. D.

Approved by:

Harold J. Reitan

D. A. Wells

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STUDIES OF A SOFT X-RAY MUTANT OF *ASPERGILLUS NIGER* VAN TIEGHEM

A dissertation submitted to the

Graduate School of Arts and Sciences
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1947

by

Violet Marion Diller

A.B. University of Cincinnati 1934

B.E. University of Cincinnati 1935

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CHAPTER I

STATEMENT OF THE PROBLEM

For a number of years, investigations have been made on the effects of various physical and chemical agents on living matter. Specific chemical compounds, radiations in all parts of the electromagnetic spectrum, and atomic particles such as electrons and neutrons have been studied in this connection. These investigations have been variously motivated and diverse in character. Among them are the research problems dealing with such topics as the effects of cosmic rays, x-rays and gamma-rays in cancer therapy, ultraviolet light in the treatment of rickets, heat as a beneficial and as a harmful agent, the influence of the atomic bomb radiations upon living matter, visible light in connection with plant growth, diathermy, radioactive isotopes in the treatment of disease, and the effect of chemical compounds on the growth and well-being of organisms. An interesting line of study which has been pursued with increasing frequency in recent years deals with the investigation of mutations induced by the deliberate application of these physical and chemical agents to organisms.

Both spontaneous and induced mutations have been studied by geneticists, biochemists, and taxonomists in order to determine the morphological and metabolic changes which have taken place and the mode of transmission of these charac-

teristics to future generations of the mutated plant or animal. Beginning with the work of Mendel (1), de Vries (2), Correns (3), and von Tschermak (4), and expanded by the technique of x-ray-induced mutations in Drosophila melanogaster developed by Muller (5), the purely genetical approach has continued to gain in importance. On the other hand, the developments in biochemistry have added immensely to the data concerned with metabolism and the myriad of chemical compounds involved in that process.

Beadle and his coworkers have combined the techniques of genetics and biochemistry into the type of research he calls "biochemical genetics" which involves the study of "... genes from the standpoint of their material composition and of their precise function in controlling developmental and functional processes." (6)

The importance of all methods of attack on the problems of inheritance and means of control of the functioning of the organism is implied in Beadle's statement about his own approach:

> If the maximum possible understanding of what the organism is and what it does is to be obtained, it is clear that our approach must be made from many sides. In the present state of knowledge the chemist cannot understand what the organism does chemically without considering genes. Therefore, the methods of genetics, which are biological - not chemical, must supplement those of chemistry. In the same way, a biologist would be blind if he were to ignore chemistry in his attempts to understand how the organism is built and how it functions. Biochemical genetics represents an approach in which the cooperation of two disciplines is essential. (6)

Whether the emphasis is biological or chemical in nature, micro-organisms are very suitable for laboratory use in problems of heredity. The difficulties and expense involved in the employment of higher animals are obvious. The higher plants are more easily handled but they also present difficulties because of the facilities which must be provided for growth. On the other hand, micro-organisms have distinct advantages but are not all equally suitable. If one is more interested in animal than in plant metabolism, the algae would be ruled out because they possess chlorophyll and engage in photosynthesis. Bacteria and the true fungi (molds), with no chlorophyll, have metabolic systems which in many respects simulate those of animals and would thus be of interest to biochemists who would wish to extend and apply any new facts to the functioning of higher animals including man. Of the two, bacteria and molds, the molds seem to be preferable. Reproduction is more complex; in many of them, the sexual phase can be used for genetical work. If one is interested in the chemistry of the organism, growth is fast and one can obtain large amounts of material for analysis, grown under controlled conditions, in a relatively short space of time.

For the purposes of this thesis, it is proposed that a culture of Aspergillus niger van Tieghem be subjected to soft x-rays, that a "mutant" differing in appearance from the normal mold be chosen at random, and that both strains,

the normal and the "mutant", be subjected to the same tests with an idea of making some comparisons in respect to fermentation characteristics, metabolism, and chemical composition.

The definition of a "mutant" as given by Thom and Raper will be used as a basis for this work:

> The term mutant, or mutation, is used to designate a strain whose source is actually known and can be verified. Furthermore, it is limited to those substrains which originated as sharp breaks from parent cultures (usually interpreted as gene mutations), and in successive culture generations retain their distinguishing characteristics unaltered. This may or may not have a taxonomic connotation.... The term mutation, then, refers to altered strains of constant character and known lineage. (7)

CHAPTER II

PRODUCTION OF THE MUTANT

X-Rays and Their Effect on Biological Material

X-rays are produced when high speed electrons impinge on a solid target. According to classical theory, x-rays are regarded as electromagnetic waves traveling through space with a velocity equal to $3\ (10)^{10}$ centimeters per second. This concept gives an explanation for such behavior as diffraction, interference, reflection, refraction, and polarization which is identical in character to that given for visible light when it is subjected to the same conditions. For a complete explanation of scattering, however, it is necessary to employ the quantum theory which maintains that "x-rays are not only emitted and absorbed as quanta but are also quanta as they travel through space. These discrete quanta of light or x-rays are ... known as photons."(8)

According to classical theory, the wave length of all of the scattered radiation should be the same as that of the incident radiation. It has been found, however, that in all directions except the forward one there is some scattered radiation with a wave length longer than that of the incident beam. An explanation of this was given by Compton according to the quantum theory (9). On this basis, a photon of energy upon striking a free electron at rest is scattered in one

direction while the electron recoils in another. In order that energy be conserved, the frequency of the scattered photon must be lower than that of the incident photon.

There is absorption of x-rays by all material through which they pass, and this means that energy is given up by the x-ray radiation. Stranathan states the following concerning this absorption:

> There are two distinct ways in which a material removes energy from an x-ray beam. One of these is by scattering the energy; the other is by true atomic absorption.... When true absorption takes place the energy given up by the x-ray is taken up by the absorbing atom. The atom absorbs this energy by having one of its electrons displaced from its normal level to some outer level, or by having this electron completely removed from the atom.... In the case of gases one sees direct evidence in cloud chamber photographs that these electrons are ejected completely from the atoms as high speed photoelectrons. The additional energy possessed by the photoelectron after being torn from the atom is gradually dissipated through the formation of a multitude of low energy ions along its path....
>
> If the concept of scattering with the consequent appearance of recoil electrons is correct, and if the concept of absorption with the consequent ejection of photoelectrons is correct, then one should expect to find one recoil electron for each quantum of energy scattered and one photoelectron for each quantum of energy absorbed. The ratio of the number of photoelectrons to the number of recoil electrons should be equal to the ratio of the true absorption coefficient to the scattering coefficient. Many experiments show that this is true within the rather large error associated with the measurements. (10)

These concepts concerning the nature of x-rays play a part in the ideas which have been advanced to explain the effect of x-rays upon living matter. The theory has been advanced that "the biological effects are due, not to the irradiations themselves, but to the shower of photo- and

Compton recoil electrons to which they give rise and to ionization which these electrons produce." (11)

A more complete analysis of the biological effects of x-rays is given by Henshaw:

> When the quantum of energy absorbed corresponds to that of x-rays or gamma rays (10,000 - 1,500,000 electron volts), an electron may be ejected from any of the orbital shells of the atom.... Since electrons in the inner shells of atoms are more firmly held than those in outer shells, a large amount of kinetic energy must be imparted to an electron of an inner orbit in causing its removal. Most important from the standpoint of the ultimate biologic effect are the facts that absorption of a high energy quantum of electromagnetic radiation causes electrons (recoil) to move through matter with initially high kinetic energy and that this energy is lost along the electron paths in production of a multiplicity of ion pairs. The number of such ion pairs per unit length of path, specific ionization, increases as the kinetic energy of the electron diminishes.
>
> In some cases, however, the full energy of the quantum is not utilized in ejection of an electron from an atom, and the residual energy is transformed into a new photon or quantum of lower energy, Compton effect. The new photon, in turn, produces another ion pair and undergoes repeated transformation until all of the energy is completely used up in production of ion pairs or thermal changes. Thus this degradation process resulting from absorption of high energy quanta causes production of vast numbers of ion pairs in the matter acted on by the radiation.
>
> When electrons are removed from the inner orbits of atoms, the vacancies are filled by electrons dropping in from outer shells, the consequence being, so far as atomic changes are concerned, a reduced number of valence electrons. Thus such a change will reduce the stability of an atom or molecule and probably result in chemical change.... this destruction of organized protoplasmic constituents and the formation of new products in living objects are responsible for radiobiologic change. (12)

Beadle states that "x-rays and gamma-rays produce their effects through the mediation of fast electrons from atoms in the tissue. These produce ions as they pass through the cells." (13) He also makes a comparison between the bio-

logical effect of x-rays and that of ultraviolet light: "In the absorption of a quantum of ultraviolet radiation by a cell, less energy is involved than in an ionization and it seems quite clear that the effect of this type of radiation is different in other respects from that of ionizing particles." (13)

There has been discussion in the literature as to whether wave length in itself is a factor or whether dosage is more important. Muller has stated:

As to the mechanism of production of those radiation mutations which affect individual genes or narrowly circumscribed chromosome regions, the most important fact that has come to light is the dependence of any given mutation upon the change that was initiated in a single individual atom, by its ionization or other excitation. This is shown by the series of findings demonstrating a strictly proportional relationship between the frequency of the induced mutations and that of the induced ionizations, no matter how (within sufficiently wide limits) the latter are distributed in time and space, provided the stage treated and the conditions accompanying treatment be kept constant. The proportionality of the mutation frequency to the radiation dosage, as thus defined, when the dosage is changed by changing the duration of treatment, was shown by Oliver, Hanson and others of my earlier collaborators, by Timofeeff-Ressovsky, and by others. (14)

Fano and Demerec have taken the same point of view:

Concerning the dosage-effect relationships, the most important experimental finding is that the frequency of certain types of radiation-induced genetic changes is proportional to the dosage of irradiation, according to the law

$$\frac{N}{N_o} = k\ D$$

where N is the number of genetic changes occurring in N_o tests, D the dosage, k a proportionality constant. (15)

The viewpoint that wave length is also a factor in the effectiveness of the irradiation is given by Failla:

> Let us examine the ionization produced by two hypothetical beams of monochromatic radiation, the wave-length of which is very different. For the sake of definiteness, imagine two point sources of equal ("candle") power, one emitting (soft) X-rays and the other gamma rays. Per unit time each source, therefore, emits the same amount of energy in all directions.... the two sources will provide the same intensity of radiation at a given distance from either. A biological material placed at this distance (from either source) will receive the same amount of energy per unit time. The complete utilization of this amount of energy through ionization of the material produces the same number of ions in both cases. But, owing to the greater penetrating power of gamma rays, the same number of ions is distributed through a much larger depth and volume of the material than in the case of X-rays. Hence the number of ions produced per second per cubic centimeter of tissue at a given depth is much smaller in the case of the gamma-ray source, under the conditions stipulated above. It is evident, therefore, that the rate at which ions are liberated at a given point in the material depends on something more than the intensity of radiation, and that this additional factor is the quality of the radiation at the point in question. The quality determines what fraction of the total energy traversing a certain layer of the material is immediately available to produce ions in the same layer. (16)

The Soft X-Ray Tube

As the source of soft x-rays for the irradiation of the Aspergillus niger culture, a gas x-ray tube (17) for producing x-rays ranging from about 0.5 Angstrom units to 4 Angstrom units was used. This tube is equipped with a copper target and a window of cellophane and aluminum which transmits the K_α and K_β lines of copper (about 1.5 Angstroms), as well as the longer and shorter wavelengths in the continuous spectrum.

The mutant studied throughout this paper was obtained by irradiating a culture of the normal mold placed 5 centimeters from the tube window when the tube was being operated at 30 kilovolts and with a tube current of 10 milliamperes. (Exact details of the handling of the culture will be given in the section of this chapter entitled Experimental Procedure). The culture was subjected to these conditions for 15 minutes.

It is possible to arrive at an evaluation of the dosage employed by referring to the curves given by Krebs and Kersten (18) in their calibration of this x-ray tube. Reading directly from the curves it is seen that operation at 30 peak kilovolts and 10 milliamperes produces a dosage of 1650 roentgens per minute at a distance of 5 centimeters from the window.

It might be well to recall that the roentgen is defined "as the quantity of X- or gamma-radiation such that the associated corpuscular emission per 0.001293 g. of air

produces, in air, ions carrying 1 electrostatic unit of quantity of electricity of either sign." (19)

Lea discusses the modification needed to arrive at the energy absorption actually taking place in tissue rather than in air:

> For the convenience of the physical measurement the roentgen is defined in terms of energy absorption per unit volume of air. In the interpretation of experiments one is interested in the energy absorption per unit volume of tissue, which for the same incident intensity of radiation will be about 1000 times greater on account of the greater density of tissue. The actual factor varies with different wave-lengths, since the ratio of the absorption coefficients of tissue and air varies somewhat with wave-lengths. It also depends on the elementary analysis of the tissue though not on the chemical nature of the compounds in which the elements are combined. (19)

This same author (19) gives a table showing energy absorption in various tissues for one roentgen of various radiations. Following are the absorption data for wet tissue irradiated with x-rays of 1.54 Angstroms:

ergs per gram	= 86.49
electron-volts per 10^{-12} gram	= 53.99
energy units	= 0.929
ionizations per 10^{-12} gram	= 1.662

In the use of soft x-rays for the irradiation of the A. niger culture there is no assumption that this is the most efficient means of producing mutants. The problem of the work is not to determine frequency of mutation under specified conditions or dependence of frequency of mutation on wavelength and/or dosage but rather to compare a mutant

with the normal A. niger in order to determine changes which have been brought about by the irradiation.

The Culture Used

A culture of Aspergillus niger van Tieghem was received from the American Type Culture Collection in September 1944. This strain is listed by them as culture number 1004.

Sabouraud's maltose agar was used as the stock culture medium for the normal A. niger and for the mutant after it was produced. This medium was made up of 1 per cent Bacto-peptone, 4 per cent technical maltose, and 1.5 per cent Bacto-agar, sterilized at 15 pounds pressure in the autoclave for 15 minutes. The three above materials are made by Difco Laboratories, Inc., Detroit, Michigan. The incubation temperature for all the work was 37 degrees Centigrade.

Experimental Procedure

Special lids were prepared for Petri dishes in order to maintain sterile conditions during the irradiation. These were made of metal and were the same shape as the ordinary glass lids except that practically all of the upper surface in the middle of the lid was missing and a horizontal flange extended beyond the vertical sides. A circular piece of cellophane was stretched over each one and tied down with

a piece of string which was held in place by the flange. The cellophane enclosure on top allowed passage of the soft x-rays.

The modified Petri dishes were sterilized in the autoclave and about 20 ml. of melted sterilized Sabouraud's maltose agar medium poured into each one. After the agar had solidified, inoculations were made in the center of each plate by means of a loop, transfers being made from a stock slant culture of the A. niger.

Over a period of two weeks, these Petri dish cultures of various ages were placed in the path of the x-ray beam, so that the radiation would go through the cellophane cover and strike the culture. All cultures were irradiated at 5 cm. from the x-ray tube window with the tube operating at 30 KV. and 10 ma. The time of the doses varied from 10 minutes to 80 minutes.

After the irradiation, the culture was scraped with a loop and serial dilutions made onto agar plates. These dilutions were made using 10 tubes each containing 10 ml. of sterilized distilled water. The scrapings from the culture, after being put into the first tube, were distributed within the water by means of a sterile 1 ml. pipette. Then 0.5 ml. were transferred to the surface of agar medium in a sterile plate. One ml. of the suspension in the first water tube was then transferred to the second tube, and after mixing, 0.5 ml. from this tube were transferred to a second sterile plate. The process was continued for the 10 tubes and 10 plates. By

means of gentle horizontal shaking, the 0.5 ml. in each plate were distributed over the surface of the agar. The plates were incubated for seven days.

The entire process was always carried out in duplicate, except that one of the cultures was irradiated and the other was not.

The plates were inspected for isolated colonies which differed in appearance from the normal A. niger culture. All plates which resulted from dilutions from the plate cultures which had not been irradiated showed typical A. niger colonies. In the dilution plates from five of the thirteen irradiated cultures, there were found colonies which were atypical. In each case, only one such colony was chosen at random and a transfer made to a sterile slant. At this same time a transfer was made from the duplicate dilution plate resulting from the culture which had not been irradiated.

Of the five mutant strains, one was chosen at random to be used for extensive analysis. The irradiation data for this mutant had been:

Age of culture at time of irradiation. .	90 hours
Distance from window of x-ray tube . .	5 centimeters
Tube voltage	30 kilovolts
Tube current	10 milliamperes
Time of exposure	15 minutes
Time interval between irradiation and dilution	6 hours

In order to insure a pure culture of the mutant strain, a plating-out method similar to that described by Smith (20) was used. Scrapings from a slant culture of the mutant were transferred by means of a loop to a tube of sterile distilled water. Serial dilutions were made and plating-out done onto agar medium in Petri dishes as described previously. The plates were incubated and it was found that after 48 hours, when the surface of the agar was viewed under a low-power binocular microscope, the spores had germinated sufficiently to put out the primary germ-tubes. A dilution plate was chosen which contained a small number of such germinated spores which were well isolated from each other. A needle with its end flattened was used to cut out small blocks of agar, each containing a single germinated spore. Each block of agar was transferred to a sterile slant. The usual flaming of needle and tubes was done.

When the slants were inspected after incubation, it was found that they all appeared to be alike and to be the same as the original mutant culture, so one was chosen at random to be the first stock culture of the mutant.

The normal culture was subjected to the entire purification process as detailed above, not because it was thought to be an impure culture but to continue the policy of subjecting both the normal and mutant strains to the same procedures.

CHAPTER III

GENERAL CHARACTERISTICS OF THE NORMAL A. NIGER AND MUTANT STRAINS

The normal culture has the typical appearance of A. niger v. Tiegh. (Fig. 1) as described by Thom and Raper (7). Colony growth is rapid and conidia are brownish-black to black in color

The mutant differs in several respects from the normal mold (Fig. 2). The sporulation is definitely depressed, to such an extent that there are patches of mycelial growth which show very few spores or none at all. The conidia are very light cream to tan in color. Viewed from the underside, a slant or plate culture is yellow-tan in color.

Under the microscope, typical Aspergillus conidial heads are seen in both normal and mutant strains. The photomicrographs shown in Figs. 3 and 4 were taken using slide-cultures made by a technique similar to that described by Henrici (21). An ordinary glass slide (3 x 1 in.) was used as a basis. Upon it was placed a drop of sterile liquid medium which was inoculated with a fine needle. A 22 x 22 mm. cover slip was placed on each end of the slide to act as support for a 22 x 40 mm. cover slip placed over the drop. Just before use the slide and cover slips had been cleaned and sterilized by means of alcohol and flaming. The prepared slide was placed in a sterile Petri dish, the bottom of

which was covered with a piece of moist blotter, and was kept there during incubation. By the use of such a slide-culture it is possible to take photomicrographs of the aerial growth found in the space around the drop of medium.

Fig. 1

Normal A. niger Growing on Sabouraud's Medium.

Single spore inoculation, photographed at 9 days.

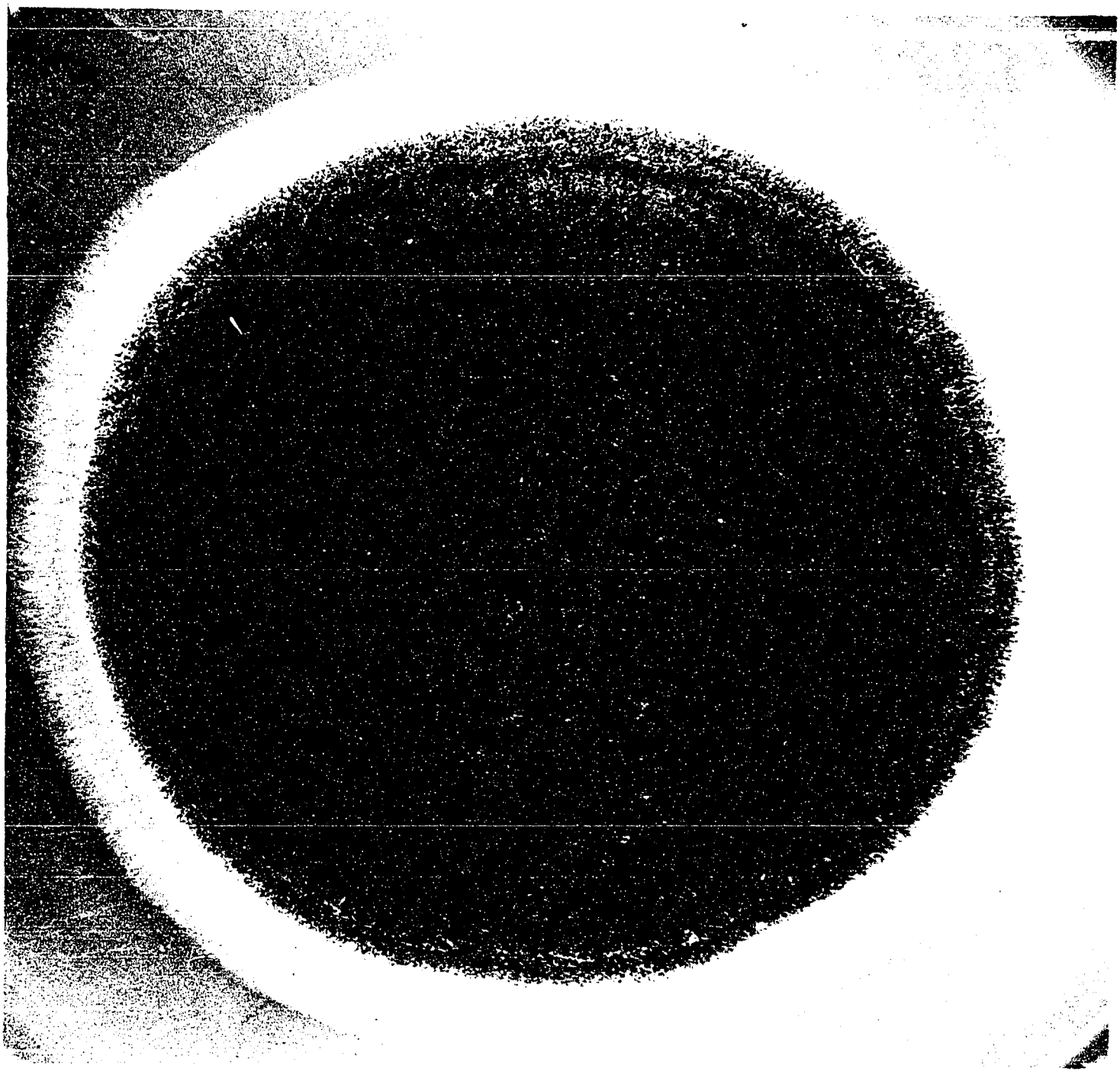


Fig. 1

Fig. 2

Mutant *A. niger* Growing on Sabouraud's Medium.

Single spore inoculation, photographed at 9 days.

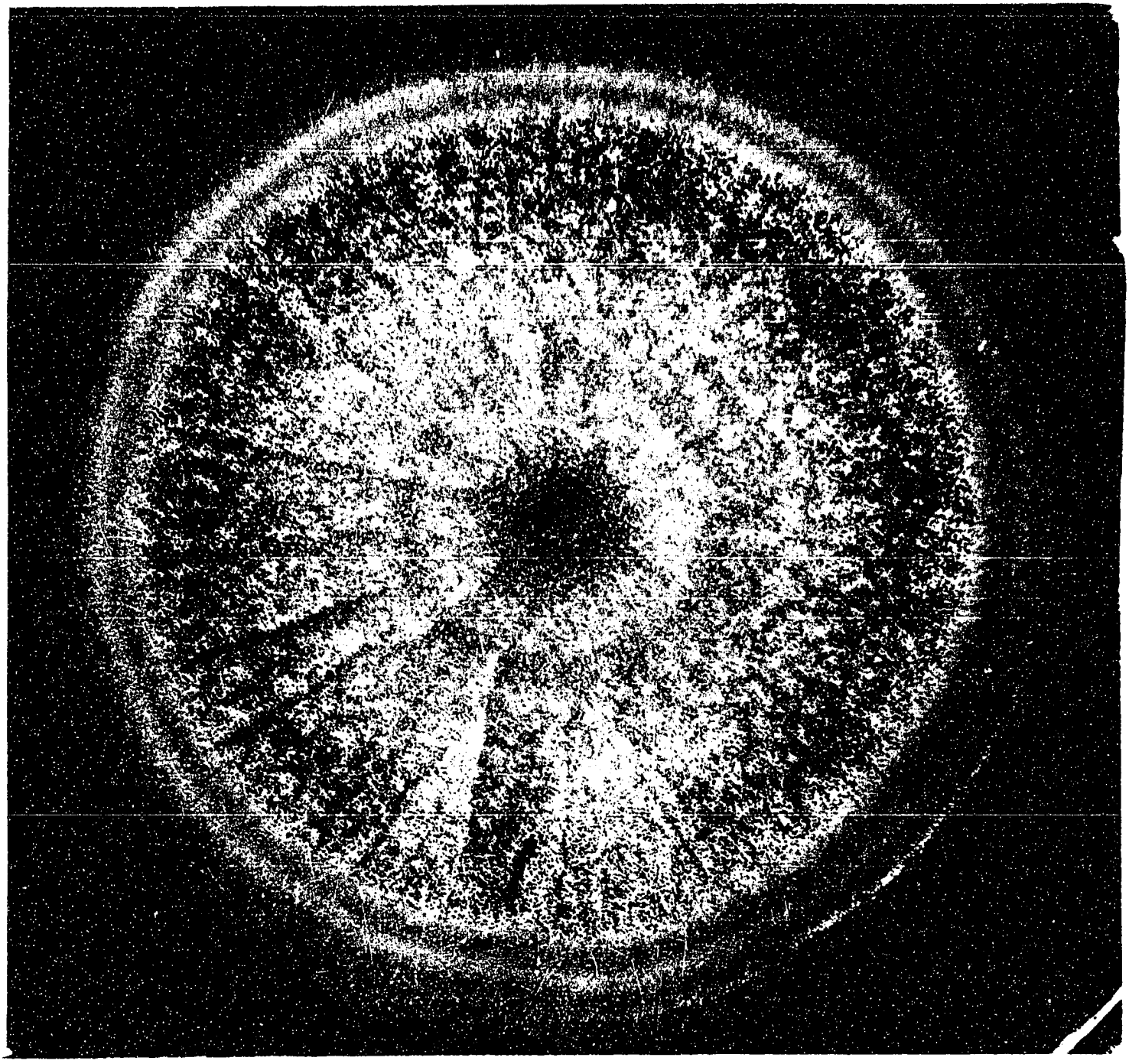


Fig. 2

Fig. 3

Photomicrograph, A. niger v. Tiegh., x 1000

Fig. 3

Fig. 4

Photomicrograph, mutant *A. niger* v. Tiegh., x 1000

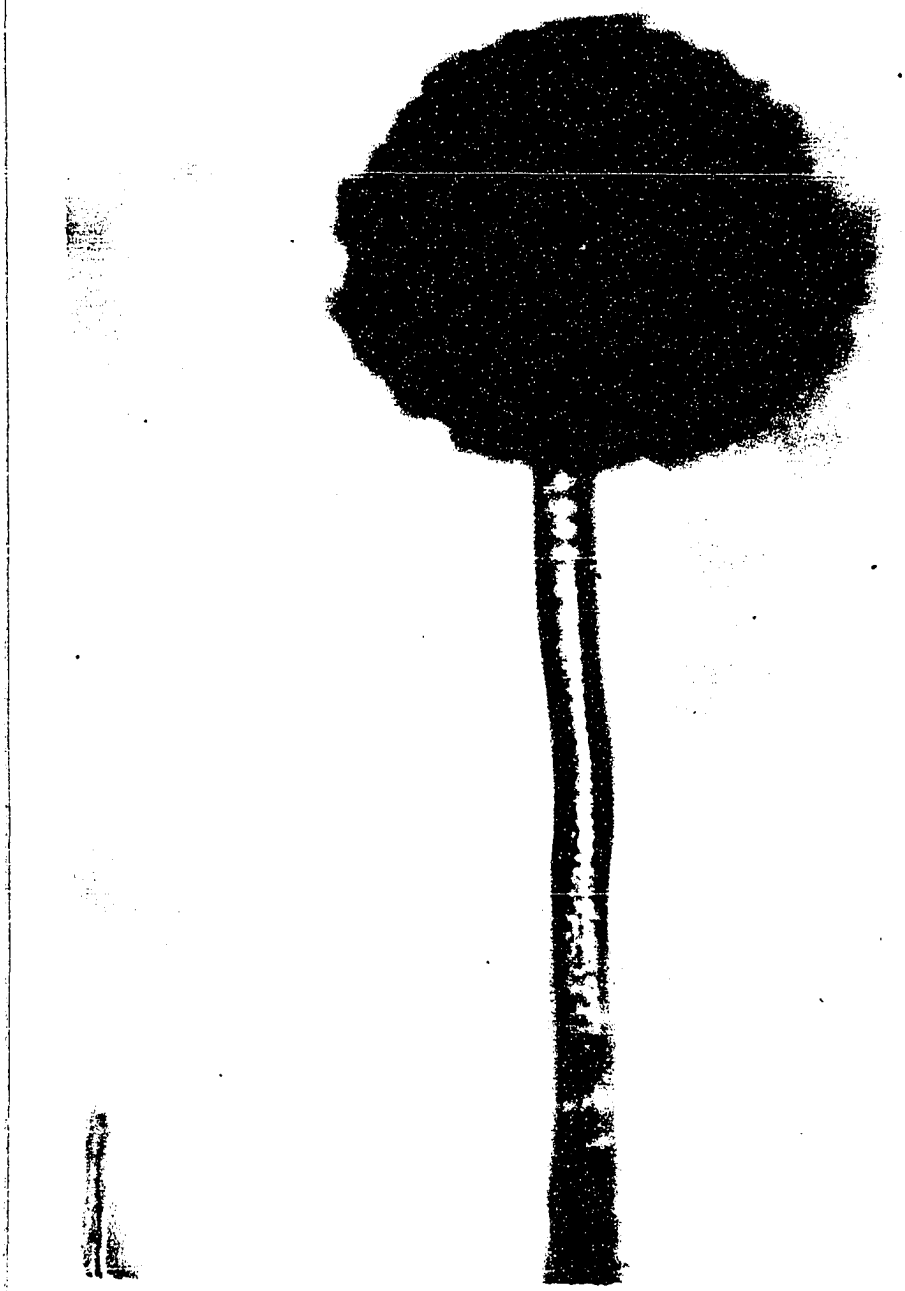


Fig. 4

CHAPTER IV

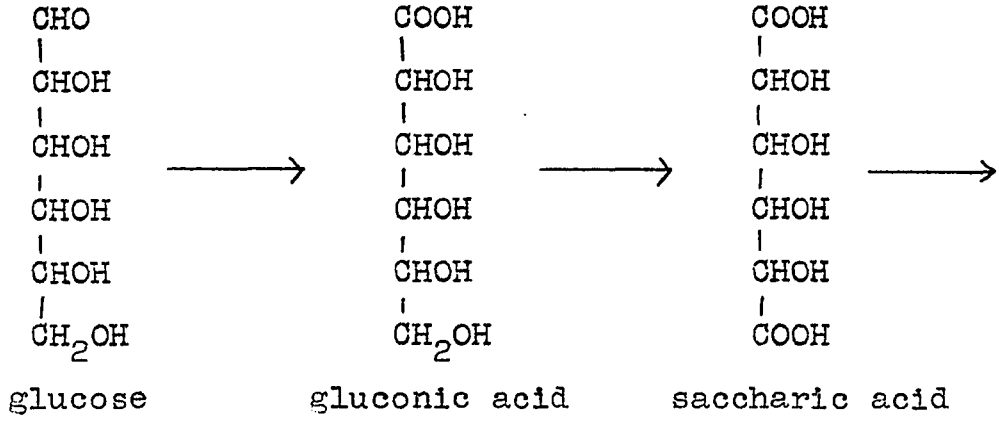
CITRIC ACID PRODUCTION CORRELATED WITH GROWTH AND SUGAR UTILIZATION

Mechanism of Citric Acid Production by Fungi

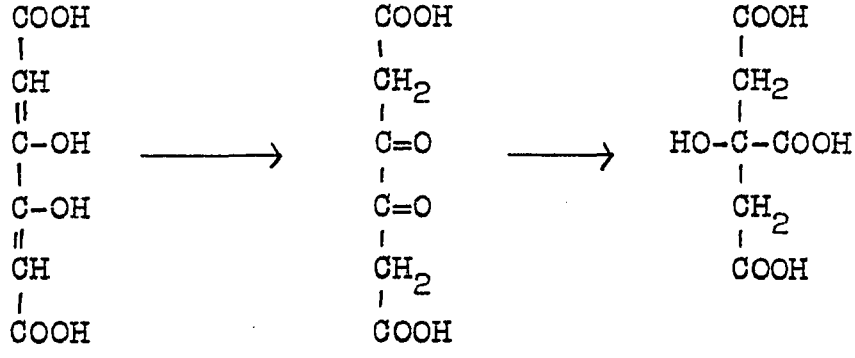
Since 1923, the mycological method for the commercial production of citric acid in the United States has steadily increased in importance (22). Currie's paper in 1917 (23) was fundamental in establishing the use of A. niger cultures for this industrial fermentation process.

Although there has been much work done on the mechanism of the citric acid production by fungi, "the true mechanism... is still obscure, and no hypothesis presented to date satisfactorily explains all of the observed facts." (22) Porter points out that the various explanations for the conversion of carbon-containing compounds to citric acid have centered around two points. "The first involves a series of reactions in which the glucose chain is not broken into simpler units but merely becomes transformed in some way to citric acid with its branched chain. The second involves the breakdown of the sugar molecule into simpler compounds, such as acetaldehyde or acetic acid, which are then used to synthesize citric acid." (24)

The first point of view suggests that glucose passes through gluconic and saccharic acids in the conversion to citric acid.



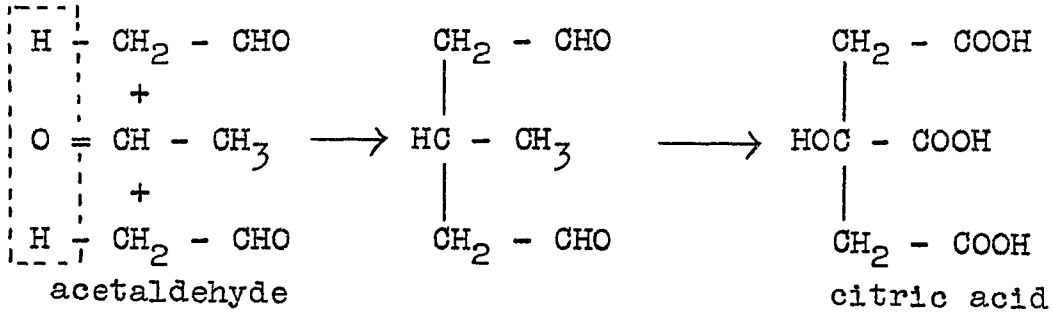
glucose gluconic acid saccharic acid



β-γ-diketoadipic acid citric acid

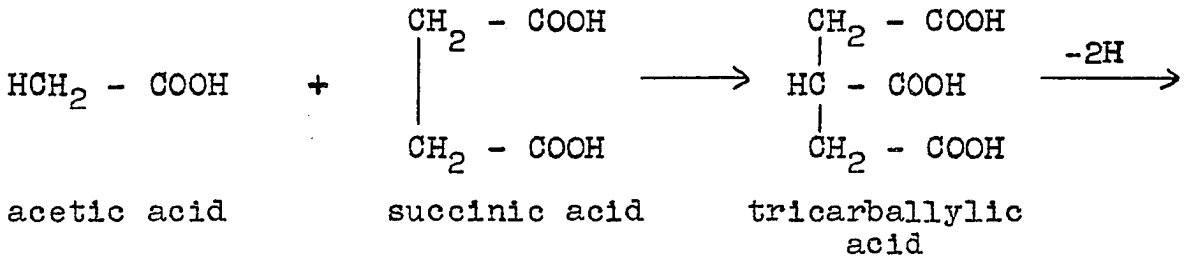
The second point of view assumes that glucose breaks down to three-carbon atoms which reunite to form citric acid. There have been three types of reactions proposed:

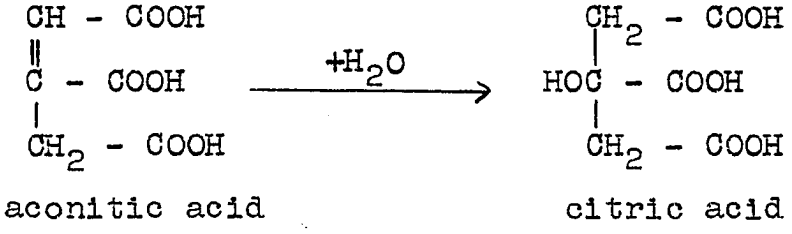
- (1) condensation of three molecules of acetaldehyde followed by oxidation



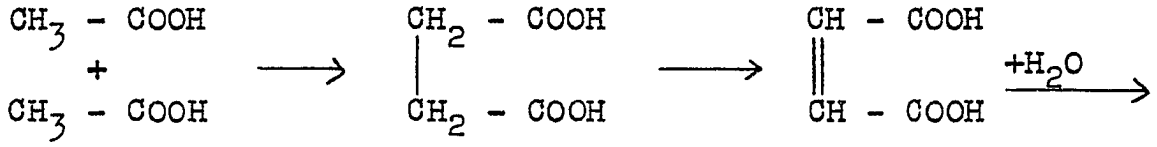
acetaldehyde citric acid

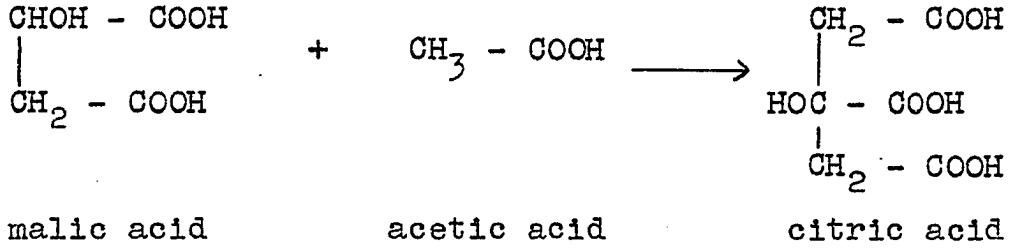
(2) union of acetic and succinic acids to form tricarballyic acid, followed by oxidation to aconitic acid which is then hydrated





(3) union of two molecules of acetic acid to form succinic acid, followed by oxidation to fumaric acid which is hydrated to form malic acid. Malic and acetic acids combine to give citric acid.





Since citric acid is the major metabolic product of A. niger, it was decided to compare the mutant and normal strains in this respect, at the same time correlating the citric acid synthesis with mat production and sugar utilization. There was to be no attempt to find optimum conditions for citric acid production but rather an investigation of the results obtained when both strains were subjected to identical conditions.

General Procedure

Fifty milliliters of liquid medium were put into each of 100 wide-mouth bottles (8 oz.) having cotton plugs. Single-spore inoculations of the mutant were made in half of the bottles and of the normal strain in the other half. The incubator, at a temperature of 37 degrees Centigrade, was provided with racks so that the bottles could be stored in a slanting position. Beginning with the second day of incubation, five bottles of each of the two strains were removed daily for determination of dried mat weight and chemical analysis for glucose and citric acid in the medium. Analyses were made to include eight days of growth. The appearance of the cultures at that time is shown in Fig. 5.

Fig. 5

Growth on Liquid Medium, 8 days

Normal A. niger on the left, mutant on the right

The bottles are shown in slanting position on an incubator rack.

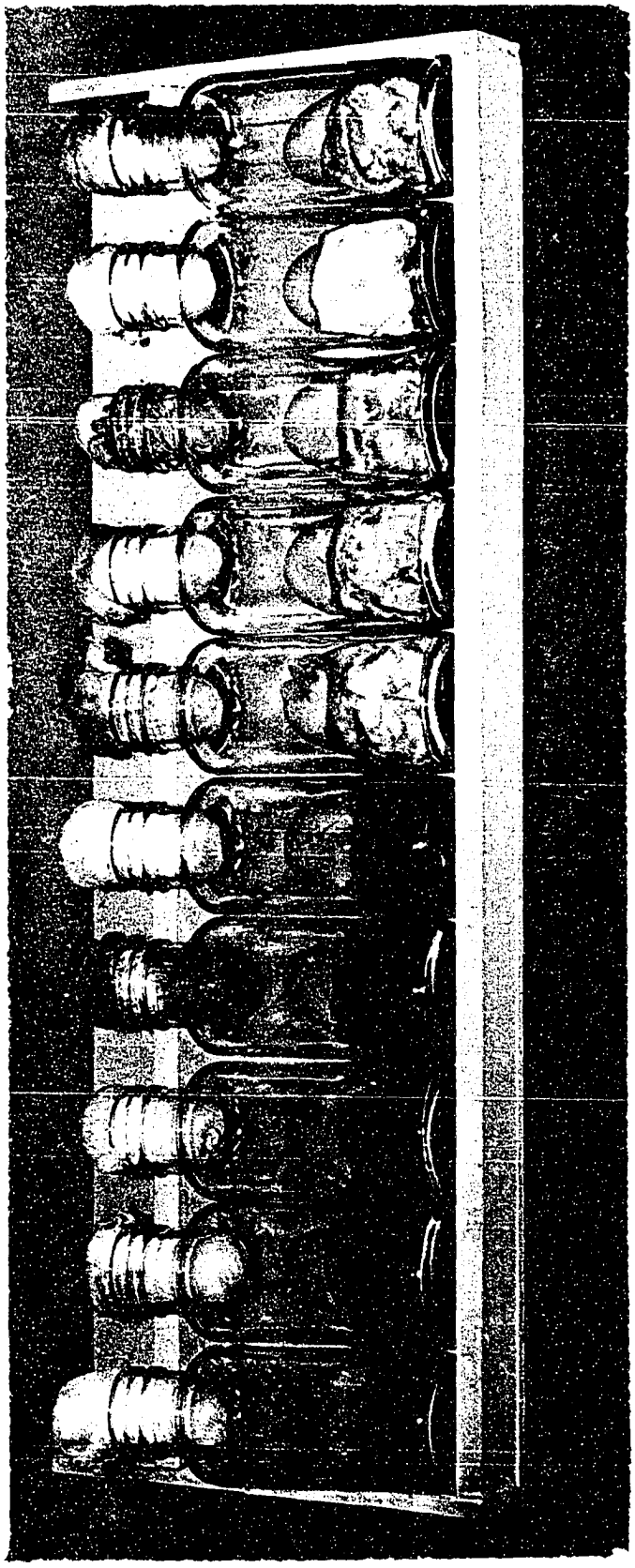


Fig. 5

Culture Medium and Preparation of Bottles

Czapek's medium (24), modified in several respects, was used. Ammonium nitrate was substituted for 20 per cent of the sodium nitrate; monopotassium phosphate was used; 0.2 per cent yeast extract was added; 10 per cent glucose was substituted for sucrose; agar was omitted; and the pH was adjusted to 3.6. Most of these changes are in line with the recommendations of Smith: "Methods were worked out for suppressing the formation of oxalic acid and increasing the yield of citric acid, the chief essentials being a high initial concentration of sugar (about 15 per cent), low concentrations of ammonium nitrate as source of nitrogen, and an acid reaction of the culture medium, pH about 3.5." (20)

The medium was made up in two solutions which were autoclaved separately.

Solution A	Solution B
3.0000 gm. $NaNO_3$	50% solution of glucose
0.7500 gm. NH_4NO_3	
1.2500 gm. KH_2PO_4	
0.6250 gm. $MgSO_4 \cdot 7H_2O$	
0.6250 gm. KCl	
0.0125 gm. $FeSO_4 \cdot 7H_2O$	
2.5000 gm. yeast extract	
distilled water up to 1 liter	
pH adjusted with HCl to 3.6	

Into each bottle, previously plugged and sterilized, 40 ml. of solution A and 10 ml. of solution B were pipetted under sterile conditions. Therefore the composition of the medium used was as follows:

2.40 gm. $NaNO_3$
0.60 gm. NH_4NO_3
1.00 gm. KH_2PO_4
0.50 gm. $MgSO_4 \cdot 7H_2O$
0.50 gm. KCl
0.01 gm. $FeSO_4 \cdot 7H_2O$
2.00 gm. yeast extract
100.00 gm. glucose
per liter of solution
pH 3.6

"Single-spore" inoculations were made in each bottle, employing the plating-out method previously described (pg. 15).

Determination of Dried Mat Weight

Gooch crucibles (size no. 4) were prepared with asbestos mat, dried at 100 degrees Centigrade for 24 hours, put into a desiccator to cool, and weighed to the nearest milligram. When the bottles were removed from the incubator, the contents were filtered, using a previously weighed crucible for each bottle. The mold mat in the crucible was

washed several times with distilled water, the washings being added to the filtrate which was set aside to be used for the chemical determination of glucose and citric acid. The crucibles, each containing the mold growth from one bottle, were dried in an oven at 100 degrees for 24 hours. After cooling in a desiccator, they were weighed and the weight of dried mat from each bottle calculated. Since five bottles of the mutant and five of the control mold were removed daily, the weights of dried mat for the five bottles were averaged.

The daily changes in dried mat weight are shown in Table 1 for the normal mold and in Table 2 for the mutant.

Table 1. Daily Change in Dried Mat Weight, Normal *A. niger*

Time (hr.)	Weight of dried mat per bottle (mg.)	Average weight of dried mat (mg.)
0.0	0	0
42.0	2 3 4 3 4	3
70.0	6 5 7 5 5	6
90.5	6 7 6 8 6	7
108.0	187 289 882 146 579	417
135.0	1277 699 934 706 907	905
156.0	845 724 876 865 900	842
180.5	939 849 966 835 933	904

Table 2. Daily Change in Dried Mat Weight, Mutant *A. niger*

Time (hr.)	Weight of dried mat per bottle (mg.)	Average weight of dried mat (mg.)
0.0	0	0
42.0	2 2 3 3 2	2
70.0	3 2 3 4 3	3
90.5	5 2 6 4 4	4
108.0	5 18 4 4 4	7
135.0	235 218 195 262 209	224
156.0	885 844 849 882 622	816
180.5	884 803 840 820 831	836

Glucose Analysis

The filtrates from the five bottles of the mutant removed daily from the incubator were combined and made up to 500 ml. with distilled water. Appropriate aliquots of this combined filtrate were analyzed for glucose. Four tests were made and the results averaged. From these average results, calculation was made of the grams of glucose per 100 ml. of filtrate, this being comparable to the grams of glucose per 50 ml. (one bottle) of the original medium. The same procedure was followed for the control mold.

The Shaffer-Hartman (25) modification of the Munson-Walker (26) determination of glucose was used. This is an iodometric method based on reduction of copper by glucose, oxidation of the cuprous ion by iodine made possible by the effect of oxalate, and titration of excess iodine with thiosulfate. The method is especially suitable for determination of glucose in the range of 20 mg. to 200 mg. The directions for the use of this method were followed as given in Browne and Zerban (27).

The following solutions are needed in the determination:

Soxhlet's Solution A (modification of Fehling's solution)

34.639 gm. $CuSO_4 \cdot 5H_2O$

in 500 ml. of solution

Soxhlet's Solution B (modification of Fehling's solution)

173 gm. Rochelle salts

(potassium sodium tartrate)

(K Na $C_4H_3O_6 \cdot 4H_2O$)

50 gm. NaOH pellets

in 500 ml. of solution

Allow to stand 2 days

Iodate-Iodide Solution

5.4 gm. KIO_3

60.0 gm. KI

Dissolve in water

Add 0.8 gm. NaOH dissolved in water

Dilute to one liter

0.1 N Thiosulfate Solution

24.822 gm. $Na_2S_2O_3 \cdot 5H_2O$

1.0 ml. $CHCl_3$ (chloroform)

in one liter of solution

Oxalate Solution

Saturated solution made by dissolving

300 gm. $K_2C_2O_4 \cdot H_2O$

in one liter of

solution

1% Starch Solution

Make paste of 0.5 gm. soluble starch and cold water.

Add boiling water and mix.

Dilute with boiling water to 50 ml.

5 N Sulfuric Acid

Dilute 133.2 ml. of conc. H_2SO_4 to one liter

Appropriate aliquots of the filtrates were chosen so that the sample contained approximately 200 mg. of glucose. Twenty five ml. of Soxhlet's solution A were transferred by pipette to a 500 ml. Ehrlenmeyer flask. Twenty five ml. of solution B were added and the two mixed by gentle shaking. Then the sample and enough water to make a final volume of 100 ml. were added. The flask was heated over a Bunsen burner which had been regulated previously so that boiling started in exactly four minutes. The boiling was allowed to continue for two minutes. After quick cooling in running water to 30-35 degrees Centigrade, 50 ml. of the iodate-iodide solution were added. Next, 15-17 ml. of 5 N sulfuric acid were added from a graduate and the mixture agitated by rotating the flask. Twenty ml. of the saturated potassium oxalate solution were added and the excess iodine titrated with thiosulfate, starch being used toward the end of the titration as an indicator.

Data obtained are given in Tables 3 and 4.

Table 3. Daily Changes in Glucose Utilization, Normal *A. niger*

Time	Aliquot of filtrate used for glucose analysis	Glucose per sample	Residual glucose per bottle	Glucose used
(hr.)	(ml.)	(mg.)	(mg./50 ml)	(mg.)
0.0	4	204.0	5100	0
42.0	4	203.5	5078	22
70.0	4	203.0	5075	25
90.5	4	202.0	5050	50
108.0	5	195.0	3900	1200
135.0	6	172.0	2867	2233
156.0	10	198.0	1980	3120
180.5	15	214.0	1393	3607

Table 4. Daily Changes in Glucose Utilization, Mutant *A. niger*

Time	Aliquot of filtrate used for glucose analysis	Glucose per sample	Residual glucose per bottle	Glucose used
(hr.)	(ml.)	(mg.)	(mg./50 ml.)	(mg.)
0.0	4	204.0	5100	0
42.0	4	201.0	5025	75
70.0	4	200.0	5000	100
90.5	4	199.0	4975	125
108.0	4	199.0	4975	125
135.0	5	236.0	4720	380
156.0	10	189.0	1890	3210
180.5	10	167.0	1670	3430

Citric Acid Analysis

Citric acid analyses were made using the method of Perlman, Lardy, and Johnson (28). This is a colorimetric method which involves the oxidation of citric acid with potassium permanganate in the presence of bromine, forming pentabromoacetone. This can be measured by the color produced upon the addition of sodium sulfide. Directions for the use of this method are given by Lardy (29).

Five milliliters of the filtrates obtained from the bottles after removal of the mold mat were used for the citric acid tests. A series of standards, differing from each other by 2.5 mg., were developed at the same time. The citric acid content of a sample was designated as lying within the range of the concentrations of two successive standards. The data in Tables 5 and 6 are reported as milligrams of citric acid per bottle (50 ml. of medium) which is comparable to 100 ml. of the filtrate.

Table 5. Daily Changes in Citric Acid Production, Normal *A. niger*

Time (hr.)	Aliquot of filtrate used for citric acid analysis (ml.)	Citric acid per sample (mg.)	Citric acid per bottle (mg./50 ml.)
0.0	5	0 - 0	0 - 0
42.0	5	0 - 2.5	0 - 50
70.0	5	0 - 2.5	0 - 50
90.5	5	0 - 2.5	0 - 50
108.0	5	2.5 - 5.0	50 - 100
135.0	5	10.0 - 12.5	200 - 250
156.0	5	10.0 - 12.5	200 - 250
180.5	5	10.0 - 12.5	200 - 250

Table 6. Daily Changes in Citric Acid Production, Mutant *A. niger*

Time (hr.)	Aliquot of filtrate used for citric acid analysis (ml.)	Citric acid per sample (mg.)	Citric acid per bottle (mg./50 ml.)
0.0	5	0 - 0	0 - 0
42.0	5	0 - 2.5	0 - 50
70.0	5	0 - 2.5	0 - 50
90.5	5	0 - 2.5	0 - 50
108.0	5	0 - 2.5	0 - 50
135.0	5	0 - 2.5	0 - 50
156.0	5	17.5 - 20.0	350 -400
180.5	5	15.0 - 17.5	300 -350

Results

Graphs showing the time rate of mat production, citric acid production, and glucose utilization, and the correlation among mat growth, citric acid, and glucose are shown in Figures 6 - 11, inclusive.

Fig. 6

Rate of Mat Production

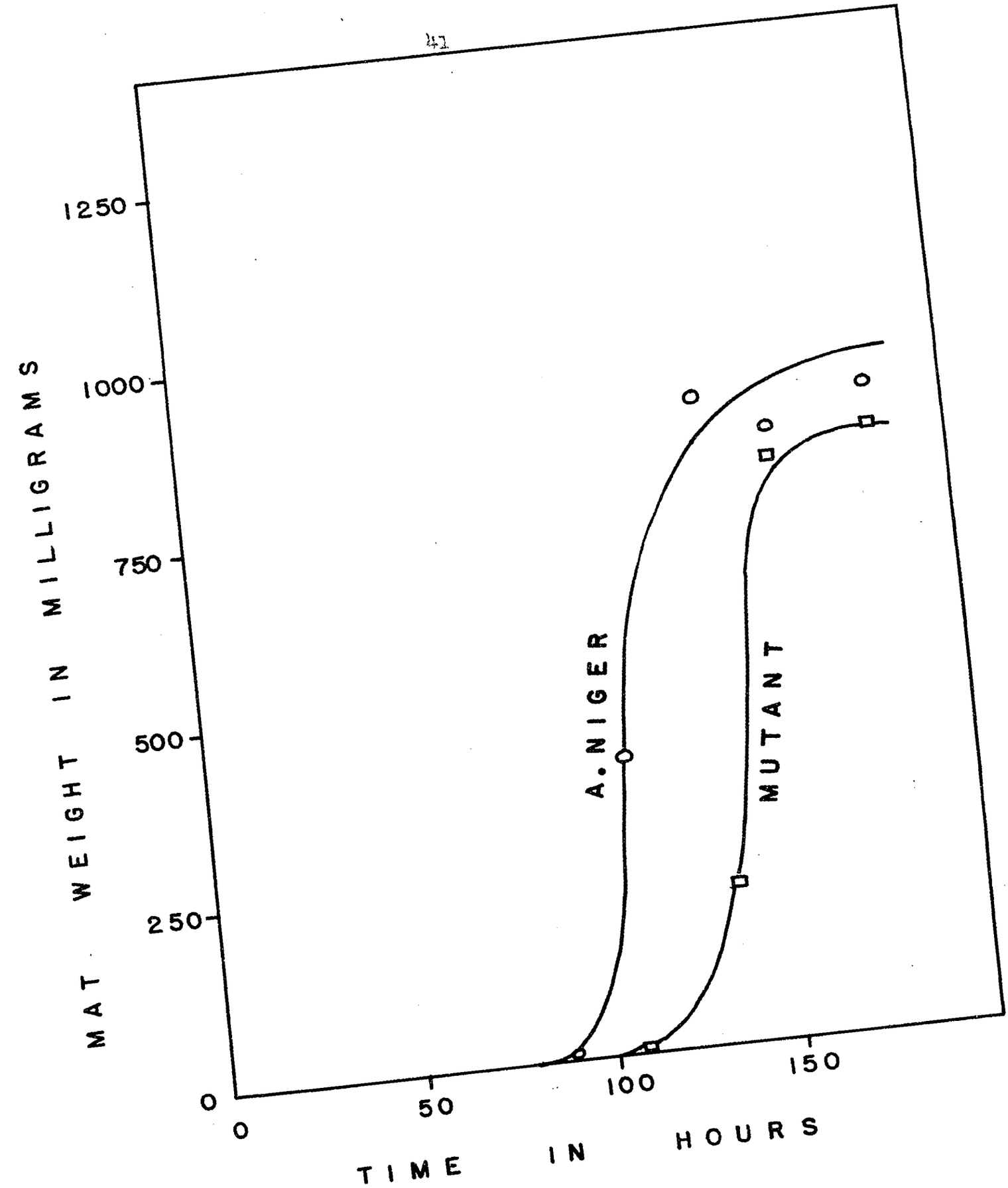


Fig. 7

Rate of Sugar Utilization

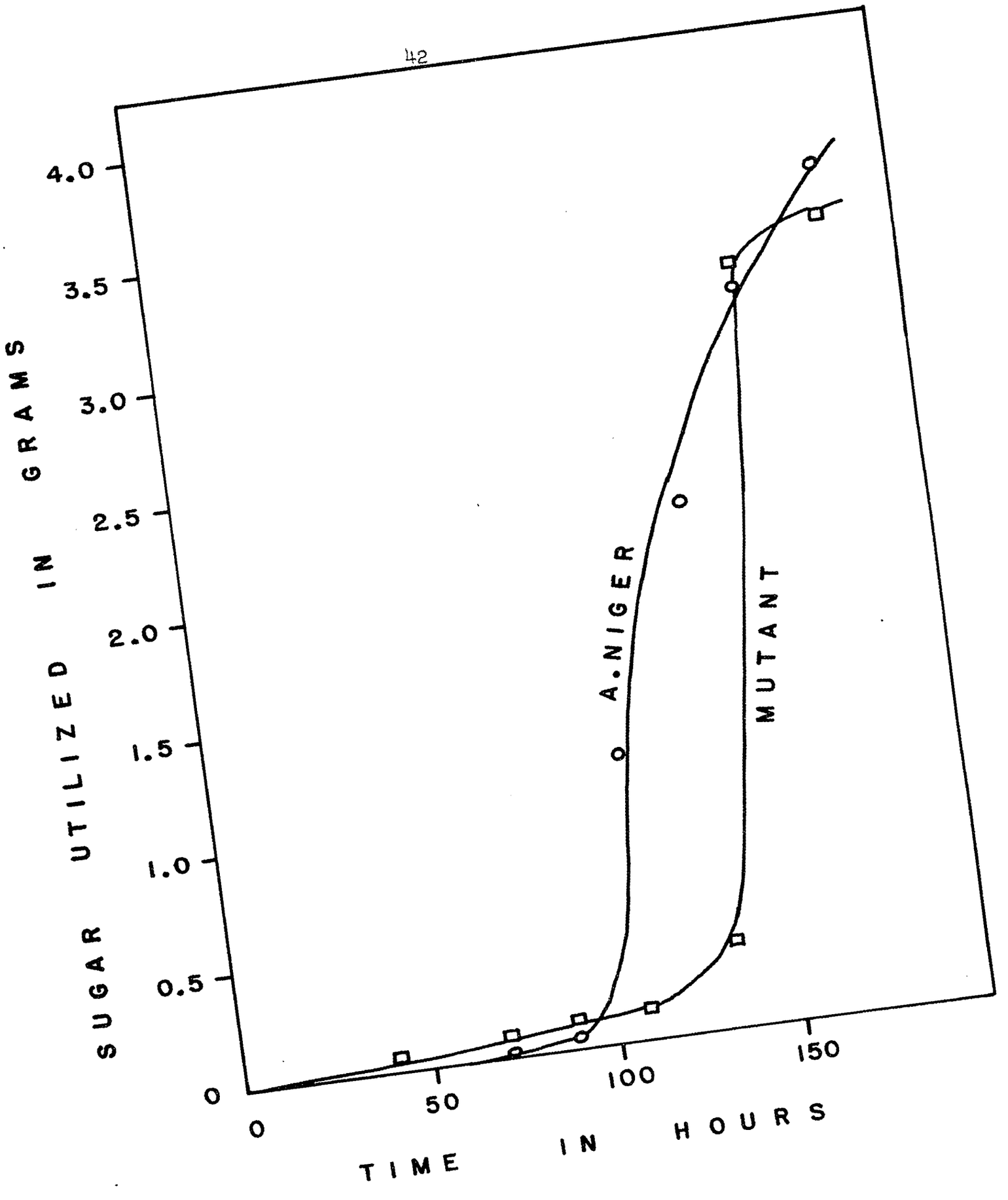


Fig. 8

Rate of Citric Acid Production

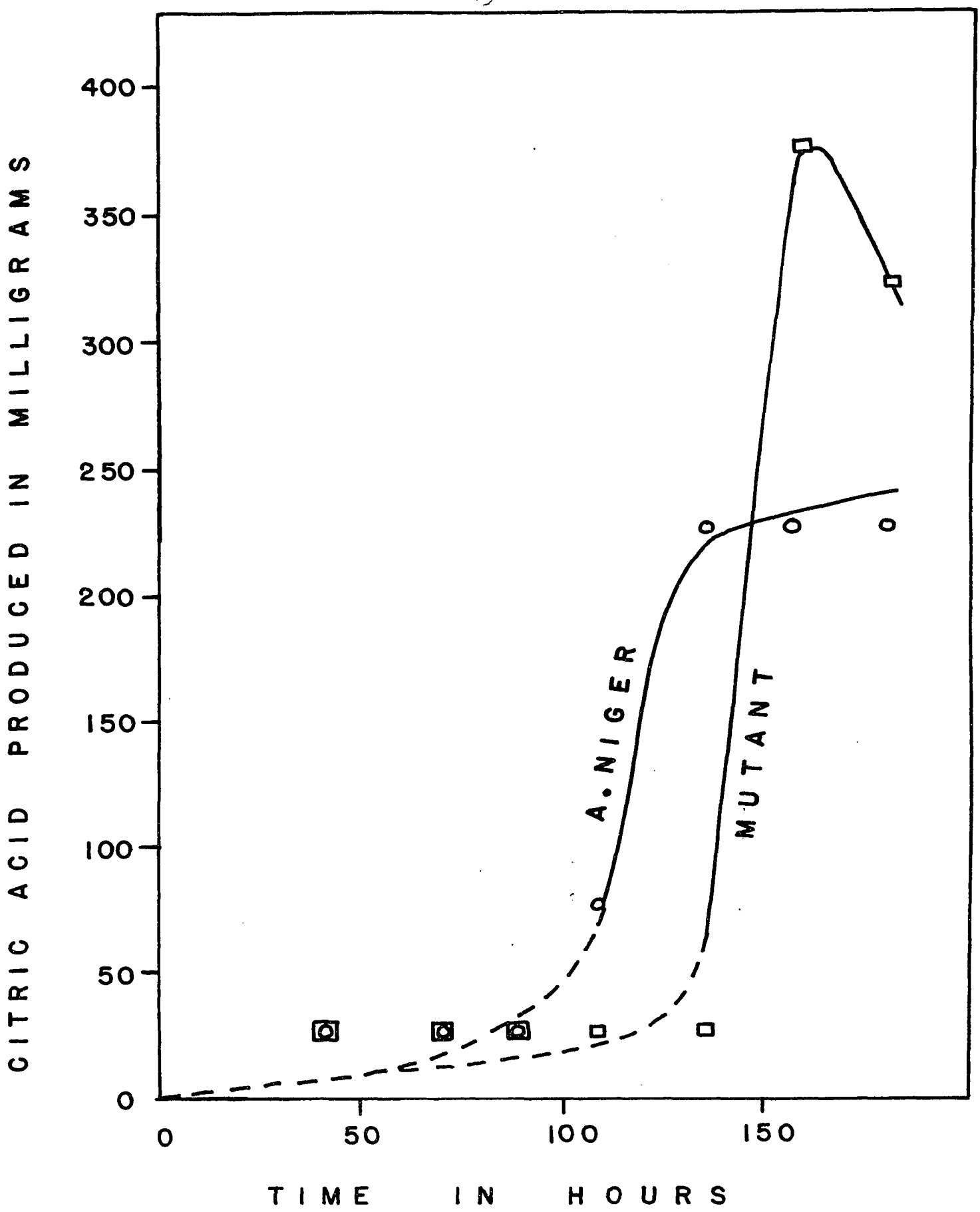


Fig. 9

Citric Acid Production vs. Sugar Utilization

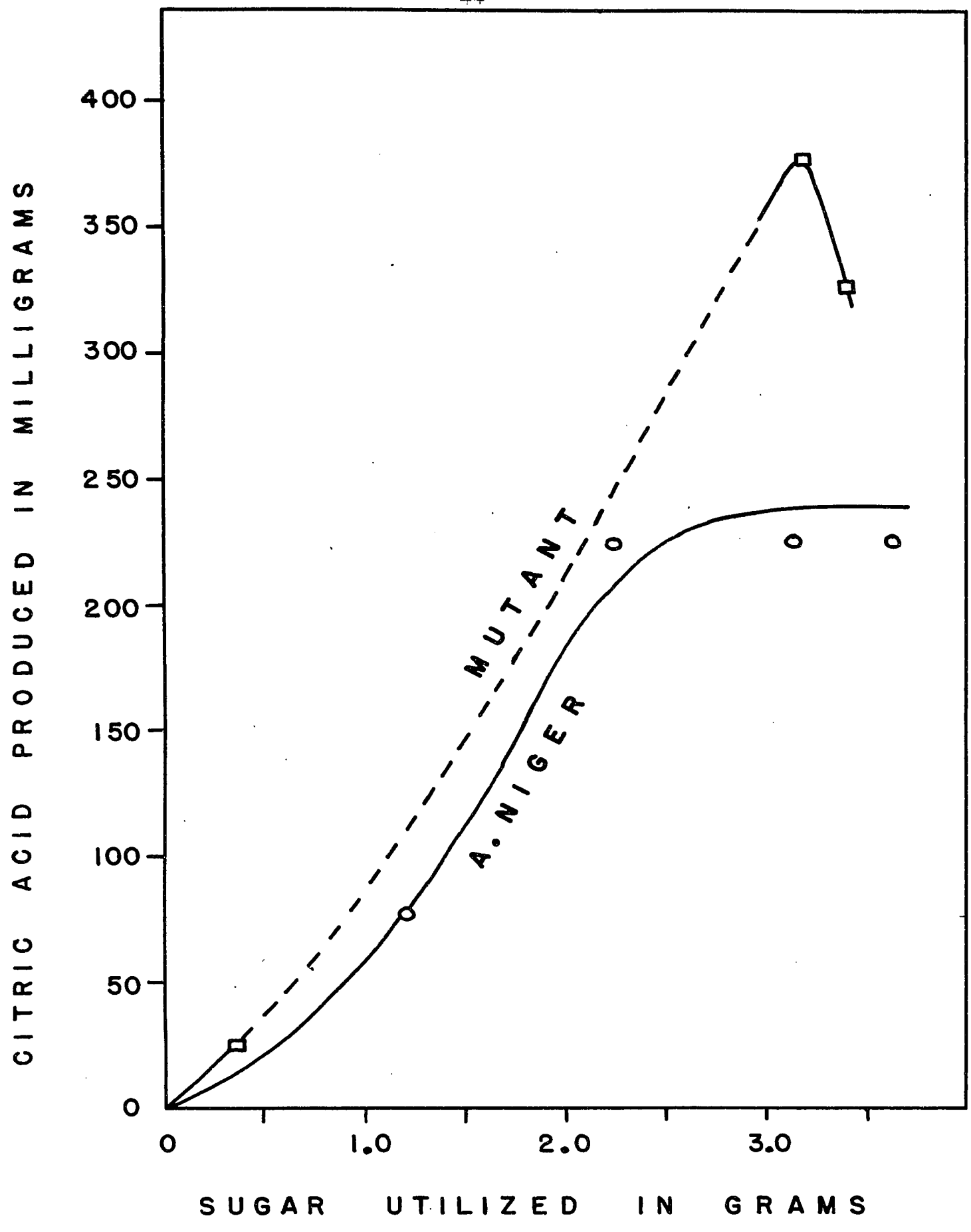


Fig. 10

Mat Weight vs. Sugar Utilization

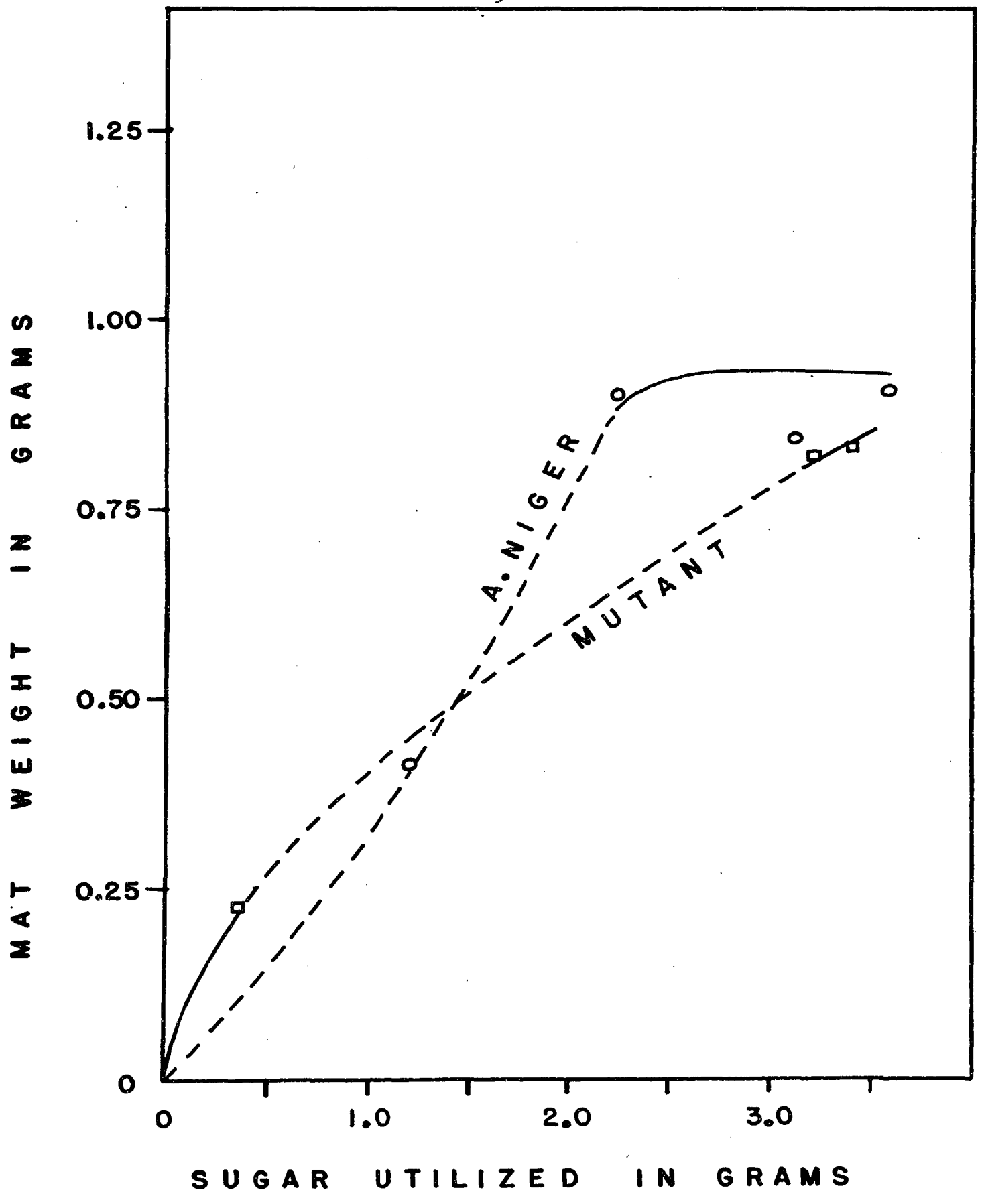
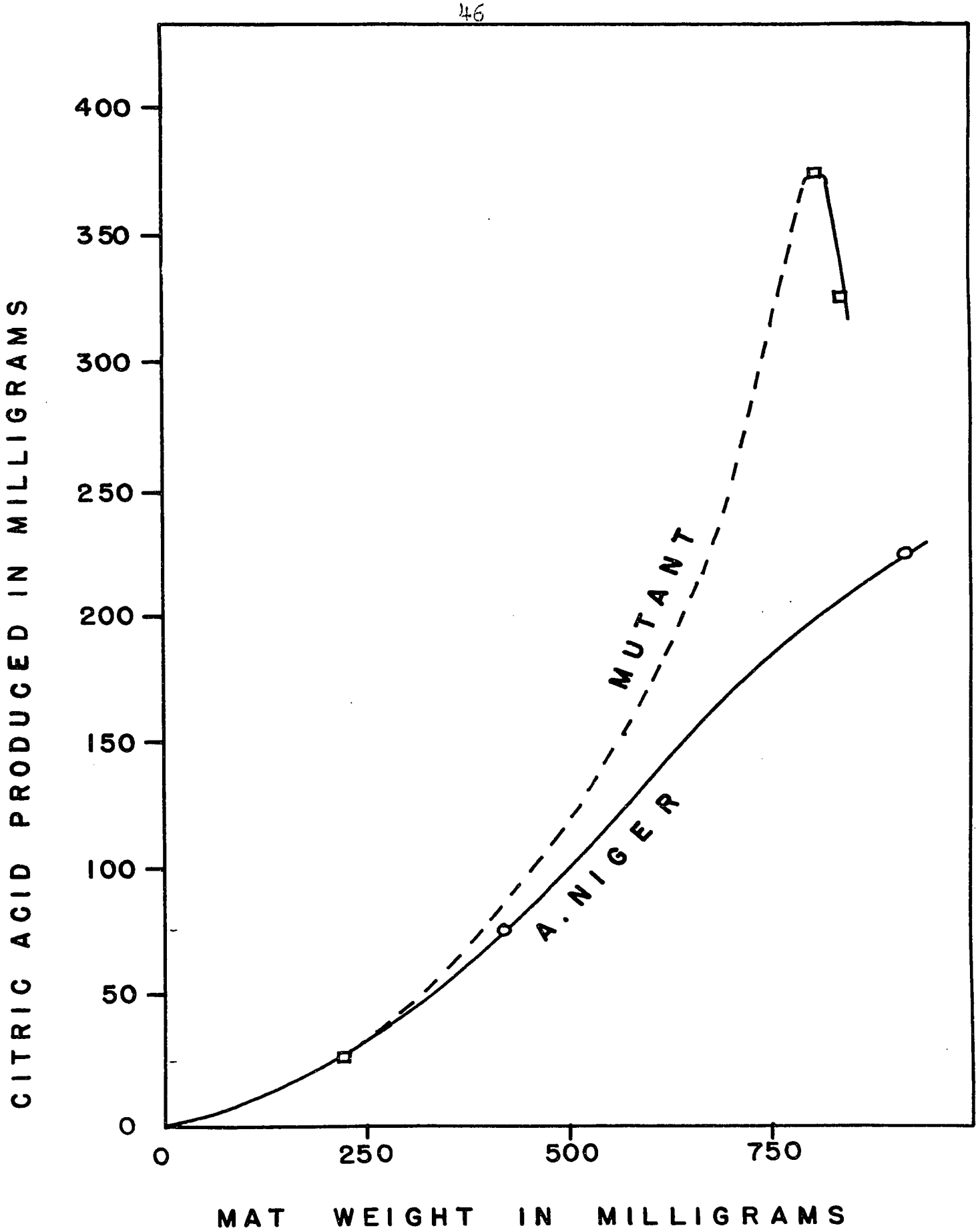


Fig. 11

Citric Acid Production vs. Mat Weight



CHAPTER V

THE EFFECTS OF GROWTH FACTORS

Introduction

There has been a large number of investigations in recent years concerned with the effect of various "growth factors" upon micro-organisms. These substances have been called by a number of names: vitamins, growth factors, accessory growth factors, growth activators, growth substances, etc. Robbins and Kavanagh give the following definition:

> A growth substance, in the specilized sense in which the term is now used, refers to a definite organic substance minute amounts of which have a materially favorable effect upon plant development. Many growth substances (perhaps all) are specific and indispensable. (30)

The same authors comment on the interpretation of the role of these substances in the growth and metabolism of an organism:

> Two general concepts of growth substances, not mutually exclusive, seem to underlie the interpretations of observations by various investigators. One concept emphasizes the relation of a given substance to the production of observable morphological changes in the organism and results in the description of root hormones, flower hormones, and hormones for cell division, cell elongation, etc. The other concept assumes that the particular growth substance plays a specific role in the metabolism of the organism and that any morphological change is incidental to this function. Growth substances probably do not all function in the same way. Many are parts of enzyme systems or precursors of such parts; some are building stones of non-enzymatic constituents of the organism, for example nucleoproteins; and it is possible that others may function in other ways, for example in maintaining surface layers or influencing permeability.*(30)

* The inclusion of this statement does not necessarily imply that the author of this thesis is in agreement.

Knight is more specific in discussing the nature of the effect of growth substances:

> ... it is seen how the aspect of the metabolic requirements of micro-organisms (and of higher animals) which first received most study was the nutritional one. On this basis the analysis of diets and nutrient media brought to light the chemical nature of many of the more elusive components of these nutrients. But emphasis on the nutritional aspect tended at first to segregate these more elusive nutrient components as a class, and general names for the class were given, such as vitamin, essential growth factor, etc. When, however, the nutritional question is viewed from the standpoint of metabolism, many of the apparently difficult questions of definition and nomenclature disappear. The substances which an organism takes from its nutrients are used as material for building-up the new cells. These cells carry out a complex interwoven series of processes, which <u>is</u> the life of those cells, and consists in taking compounds from the environment and synthesizing other compounds to make new cells. The extent and rate of multiplication of new cells will depend on the efficiency with which the processes of construction are carried out. This efficiency (here used in the general and not only thermodynamic sense) will clearly depend partly on the availability of the materials of construction. This will in turn depend upon the rates of utilization and synthesis of the various materials of the enzyme systems whose continued functioning is the life of the cells. The fundamental biochemical processes of cell-life - the essential metabolism of the cells - form the cardinal feature, and certain of these processes are common to the widest variety of cell. Where organisms may differ, however, is in the means whereby the materials for these processes are acquired. But here a sharp metaphysical distinction into "acquired from the environment" or "synthesized by the cell" is not possible. For it is clear that a certain rate of synthesis might be too slow to yield a required substance at the required rate. Effectively then the cell would depend upon an external source of supply, to a degree which would be relative to the rate of synthesis of this substance. Hence, a given substance, required as a component of one of the essential metabolic processes, might appear in three different roles as a component of the nutrients. It might appear: (1) as an essential nutrient, when its rate of synthesis by the cell was so slow as to be insignificant; (2) as a growth stimulant, when its rate of synthesis was somewhat faster but still slow enough to be a limiting factor; or, (3) as a substance not required at all for nutrition, because the cell could

synthesize it so fast that it was not a limiting factor in growth. It is the metabolic process which is the essential thing and the compounds used in carrying it out are essential metabolites, i.e., the substrates used for the process, or the substances which form parts (prosthetic groups, etc.) of the enzyme systems which carry out these essential reactions.... discussions as to whether a given substance is an essential growth factor, vitamin, etc. are clearly sterile. What matters is to show how any given substance which affects growth plays its part. And very often it will be found that it has a relation to some essential metabolic process, the role it plays in nutrition reflecting the mode by which the cell acquires a sufficient quantity of it at a sufficient rate. (31)

General Procedure

Individual growth factors were added to basic medium in order to compare their effects upon the growth of the mutant with those upon the normal mold. Single spore inoculations were made on solid medium in Petri dishes. Growth was determined by measuring the diameters of the resulting colonies and from this calculating the areas. For each growth substance, the areas were compared with those on basic medium, the two values being determined simultaneously. Incubation was at 37 degrees Centigrade for seven days.

The Basic Medium

The same concentrations of inorganic salts were used as had been employed in the glucose-citric acid experiments (pg. 28). Glucose, in a concentration of 1%, was the only other constituent of the medium. Silica gel was

used as the solidifying agent in order to avoid the use of agar and any effect it or its impurities might have upon growth (32).

To supply the constituents of the silica gel, stock solutions of sodium silicate and hydrochloric acid were made. Concentrated HCl was diluted 1:12 with distilled water and saturated Na_2SiO_3 solution was diluted until the two solutions were equivalent in titration using bromophenol blue as an indicator.

Preparation of the Petri Dishes

The inorganic salts-glucose solution used in preparing the medium was made up as follows:

2.40 gm. $NaNO_3$
0.60 gm. NH_4NO_3
1.00 gm. KH_2PO_4
0.50 gm. $MgSO_4 \cdot 7H_2O$
0.50 gm. KCl
0.01 gm. $FeSO_4 \cdot 7H_2O$
10.00 gm. glucose
per 50 ml. of solution

Into 25 x 200 mm. test tubes were put 9 ml. HCl solution, 7.3 ml. Na_2SiO_3 solution, 1 ml. of the inorganic salts-glucose solution, and 1 ml. of solution of the particular growth substance being used. Instead of the last, 1 ml. of water was put in the tubes to be used for the plates which

were to contain only basic medium. Two ml. of the Na_2SiO_3 solution were put into an equal number of 15 x 125 mm. tubes. All tubes were plugged with cotton and sterilized.

Solidified medium in Petri dishes was made by pouring the contents of a large tube and a small tube into each dry sterile plate. The two solutions were mixed immediately by revolving each plate in a horizontal plane.

By autoclaving 2 ml. of the silicate solution separately, it was possible to keep the medium in liquid form until after autoclaving. Since the HCl and Na_2SiO_3 solutions were equivalent, equal amounts of both would cause jelling, but it was found necessary to use the extra 0.3 ml. of the silicate solution in order to take care of the amount that adhered to the sides of the tube when the 2 ml. were poured out of the small tube.

Inoculation

"Single spore" inoculations were made in the center of each plate. This was done by using the serial dilution-plating out method described previously (pg. 15), the only difference being that silica gel basic medium was used for the dilution plates instead of Sabauroud's maltose agar.

Results

Table 7 gives the average change in colony area for both the normal A. niger and the mutant due to adding to the basic medium each of twenty different substances tested. Table 8 shows the effect of eight of these substances over a wider range of concentration.

A comparison of the normal mold and the mutant in respect to these nutritional requirements is summarized in Table 9. For the purposes of this table, the greatest per cent of increase in area obtained for each substance was calculated. It was then found that the substances could be divided into groups, each group producing percentages of increase in growth which fell into well-separated ranges.

Each photograph shown in Figures 12-29 was taken when the plates were removed from the incubator at 168 hours. For each concentration of growth substance, there is shown the normal mold on basic medium (upper left plate), the mutant on basic medium (lower left plate), the normal mold on medium to which the growth substance has been added (upper right plate, and the mutant on medium containing the growth substance (lower right plate). The plates chosen for the photographs were those having colony areas most nearly equal to the average area found in the experiment.

Table 7. Changes in Colony Area Due to the Addition of Individual Growth Substances to Basic Medium

Growth Factor	Concentration per plate (20 ml.)	Average area of colonies in $cm.^2$ at 168 hours: *A. niger* on basic medium	*A. niger* on medium with growth factor	Mutant on basic medium	Mutant on medium with growth factor
adenine sulfate	500 µg.	41.9	36.3	6.6	7.6
	10 µg.	41.9	34.2	6.6	5.3
p-amino-benzoic acid	500 mµg.	33.2	36.3	1.8	4.2
	5 mµg	33.2	38.5	1.8	5.3
biotin	500 mµg.	17.4	16.6	6.2	15.9
	5 mµg.	17.4	18.9	6.2	6.6
casein hydrolysate*	0.4 %	41.9	25.5	8.0	10.8
	0.3 %	41.9	34.2	8.0	13.9
	0.2 %	41.9	36.3	8.0	8.6
	0.1 %	41.9	30.2	8.0	10.8
choline chloride	500 mµg.	17.4	15.9	6.2	5.7
	5 mµg.	17.4	16.6	6.2	6.2
cytosine monohydrate	500 µg.	31.2	32.2	2.5	4.2
	10 µg.	31.2	41.9	2.5	6.6
guanine hydrochloride	500 µg.	40.7	44.2	6.6	12.0
	10 µg.	40.7	36.3	6.6	10.2
hypoxanthine	500 µg.	31.2	43.0	2.5	8.6
	10 µg.	31.2	27.4	2.5	10.2
inositol	500 mµg.	33.2	37.4	1.8	3.1
	5 mµg.	33.2	37.4	1.8	4.9
niacin	500 mµg.	27.3	24.6	5.3	8.0
	5 mµg.	27.3	28.3	5.3	9.1

*General Biochemicals, Inc. "Vitamin Free"

Table 7 (Continued)

Growth Factor	Concentration per plate (20 ml.)	Average area of colonies in cm.^{2} at 168 hours: *A. niger* on basic medium	*A. niger* on medium with growth factor	Mutant on basic medium	Mutant on medium with growth factor
pimelic acid	500 mμg.	41.9	36.3	6.6	10.2
	5 mμg.	41.9	36.3	6.6	7.6
pyridoxamine	50 μg.	26.4	29.2	5.7	10.2
	30 μg.	26.4	32.2	5.7	13.9
	10 μg.	26.4	29.2	5.7	8.0
	5 μg.	26.4	30.2	5.7	4.2
	1 μg.	26.4	31.2	5.7	6.2
pyridoxine hydrochloride	500 mμg.	34.2	43.0	3.1	4.5
	5 mμg.	34.2	38.5	3.1	4.5
riboflavin	500 mμg.	34.2	34.2	3.1	5.3
	5 mμg.	34.2	34.2	3.1	5.3
thiamin hydrochloride	500 mμg.	27.3	28.3	5.3	5.3
	5 mμg.	27.3	32.2	5.3	5.7
thymine	500 μg.	32.2	43.0	10.8	6.6
	10 μg.	32.2	40.7	10.8	12.6
uracil	500 μg.	32.2	33.2	10.8	10.8
	10 μg.	32.2	37.4	10.8	9.1
uric acid	500 μg.	31.2	44.2	2.5	8.0
	10 μg.	31.2	39.6	2.5	7.6
xanthine	500 μg.	40.7	43.0	6.6	7.6
	10 μg.	40.7	39.6	6.6	6.2
yeast extract (119 hours)	40 mg.	13.8	40.7	3.5	19.6
	20 mg.	13.8	30.2	3.5	15.2

Table 8. Change in Colony area Due to the Addition of Various Concentrations of Eight Individual Growth Substances to Basic Medium

Growth factor	Concentration per plate (20 ml.)	Average area of colonies in cm.^{2} at 168 hours: *A. niger* on basic medium	*A. niger* on medium with growth factor	Mutant on basic medium	Mutant on medium with growth factor
p-amino-benzoic acid	5 µg.	26.4	26.4	7.6	8.0
	500 mµg.	26.4	32.2	7.6	10.2
	50 mµg.	26.4	32.2	7.6	6.6
	5 mµg.	26.4	32.2	7.6	7.1
	0.5 mµg.	26.4	32.2	7.6	8.0
biotin	2.5 µg.	23.8	24.6	7.1	22.9
	1.0 µg.	23.8	34.2	7.1	24.6
	500 mµg.	23.8	39.6	7.1	22.9
	250 mµg.	23.8	32.2	7.1	18.9
	100 mµg.	23.8	32.2	7.1	22.1
	50 mµg.	23.8	31.2	7.1	21.2
	5 mµg.	23.8	31.2	7.1	18.1
	0.5 mµg.	23.8	35.3	7.1	10.8
cytosine monohydrate	200 µg.	18.1	14.5	6.6	7.1
	50 µg.	18.1	16.6	6.6	3.5
	20 µg.	18.1	15.9	6.6	5.7
	10 µg.	18.1	15.9	6.6	5.7
	1 µg.	18.1	11.3	6.6	9.6
guanine hydrochloride	200 µg.	33.2	30.2	6.6	7.1
	50 µg.	33.2	33.2	6.6	9.1
	20 µg.	33.2	33.2	6.6	8.0
	10 µg.	33.2	34.2	6.6	10.8
	1 µg.	33.2	25.5	6.6	9.6
hypoxanthine	200 µg.	26.4	24.6	3.8	6.6
	50 µg.	26.4	27.4	3.8	9.1
	20 µg.	26.4	27.4	3.8	6.6
	10 µg.	26.4	31.2	3.8	7.6
	1 µg.	26.4	33.2	3.8	7.6
niacin	5 µg.	34.2	31.2	4.9	4.2
	500 mµg.	34.2	19.6	4.9	5.3
	50 mµg.	34.2	22.1	4.9	7.1
	5 mµg.	34.2	22.9	4.9	4.9
	0.5 mµg.	34.2	21.2	4.9	5.3

Table 8 (Continued)

Growth factor	Concentration per plate (20 ml.)	Average area of colonies in cm.^{2} at 168 hours: *A. niger* on basic medium	*A. niger* on medium with growth factor	Mutant on basic medium	Mutant on medium with growth factor
thymine	200 µg.	30.2	34.7	9.1	5.3
	50 µg.	30.2	31.2	9.1	5.3
	20 µg.	30.2	30.2	9.1	7.1
	10 µg.	30.2	30.2	9.1	10.8
	1 µg.	30.2	28.3	9.1	13.2
uric acid	200 µg.	21.2	25.5	8.6	7.6
	50 µg.	21.2	23.8	8.6	7.1
	20 µg.	21.2	26.4	8.6	6.6
	10 µg.	21.2	24.6	8.6	8.6
	1 µg.	21.2	31.2	8.6	10.2

Table 9. Nutrition - Comparison of Normal *A. niger* and Mutant

Average per cent of increase in growth		
A. niger		Mutant
> 190%	yeast extract	> 460%
70%	biotin	240%
10-50%	p-aminobenzoic acid hypoxanthine inositol pyridoxamine	140-200%
< 20%	casein hydrolysate cytosine monohydrate guanine hydrochloride niacin pyridoxine hydrochloride pimelic acid riboflavin thymine	40-75%
< 20%	adenine sulfate choline chloride thiamin hydrochloride uracil	< 20%
50%	uric acid xanthine	< 20%

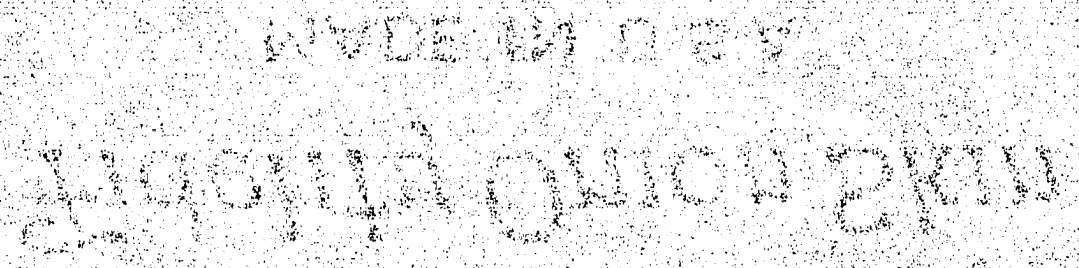
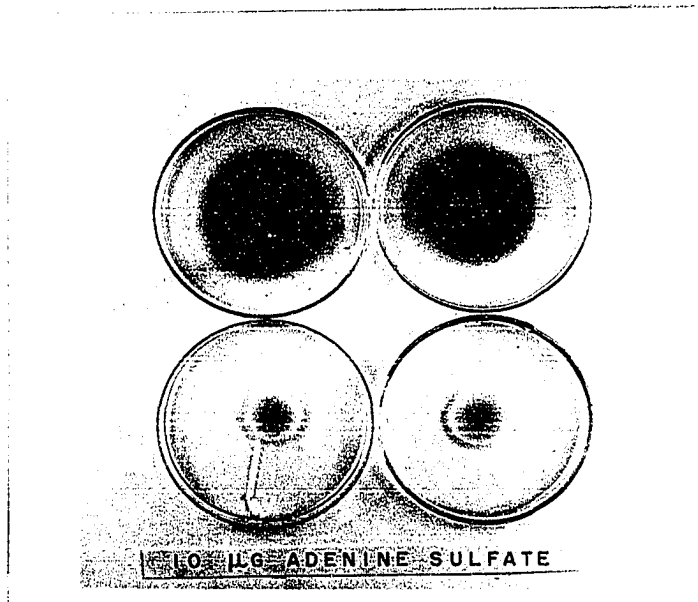


Fig. 12

Growth Changes Caused by the Addition of Adenine Sulfate to the Medium.

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



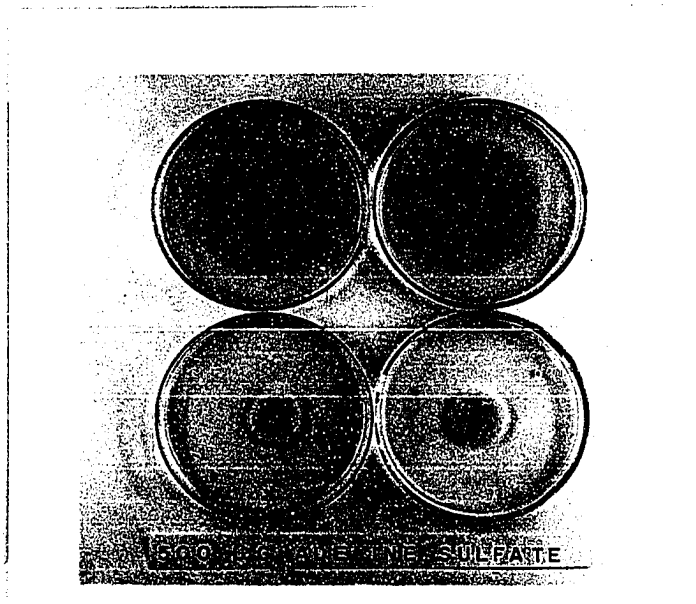


Fig. 12

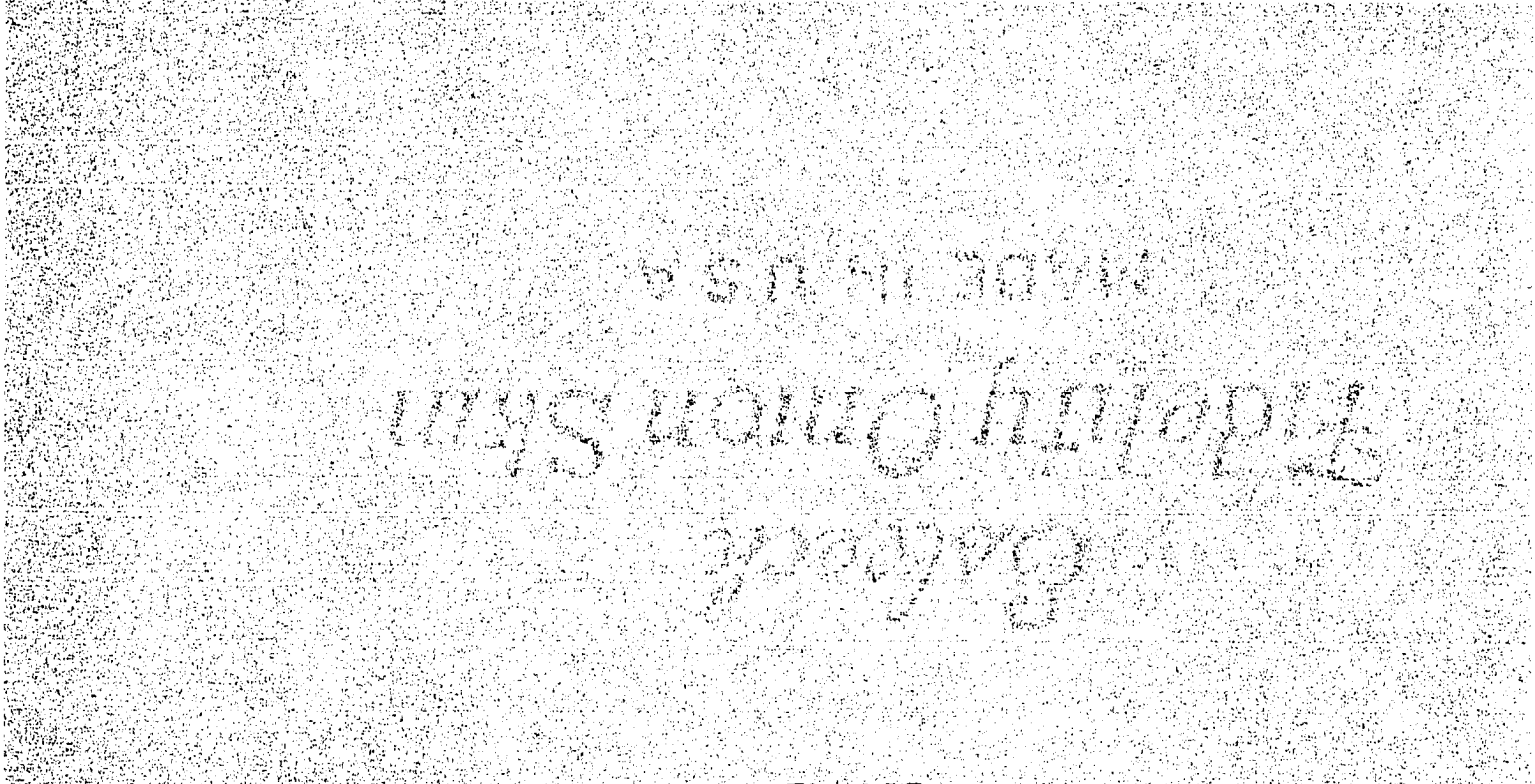
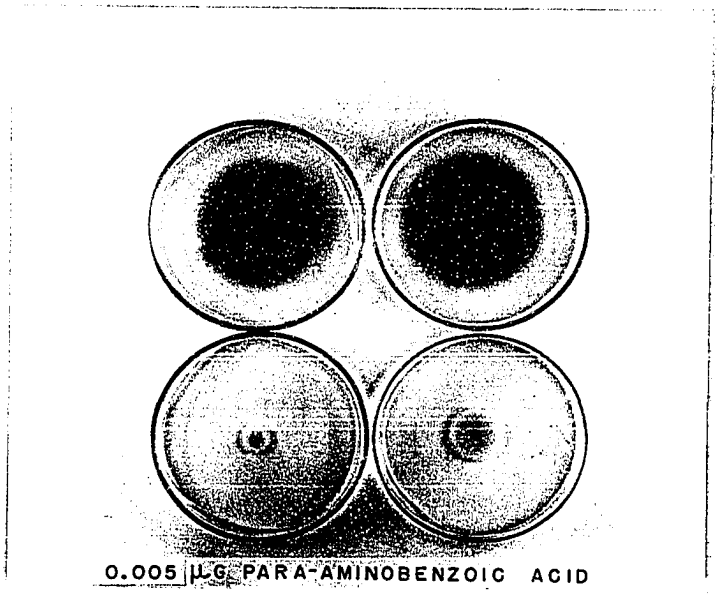


Fig. 13

Growth Changes Caused by the Addition of p-Amino-benzoic Acid to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



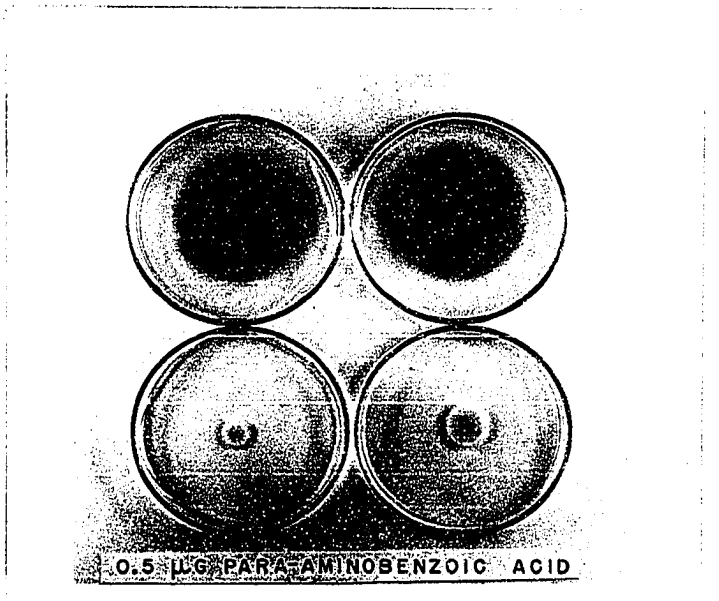
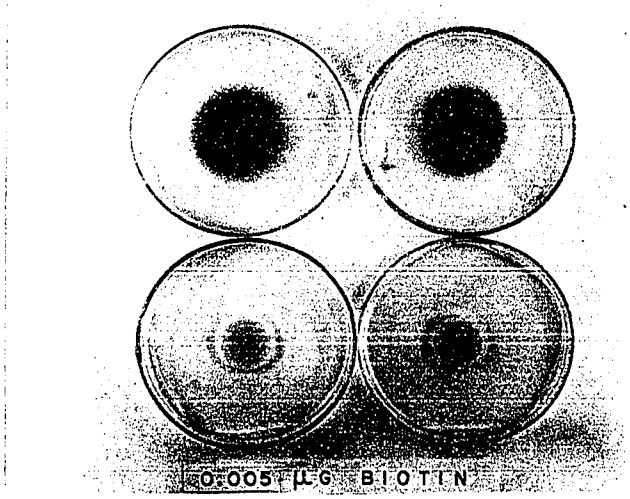


Fig. 13

Fig. 14

Growth Changes Caused by the Addition of Biotin to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



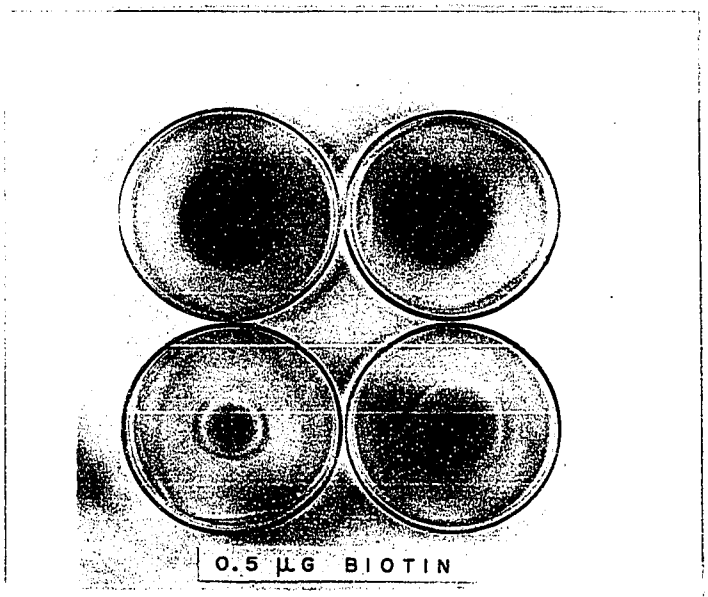
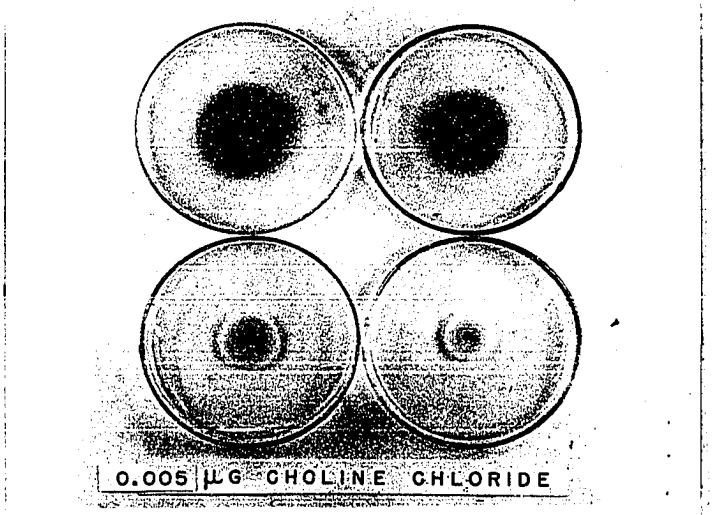


Fig. 14

Fig. 15

Growth Changes Caused by the Addition of Choline Chloride to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



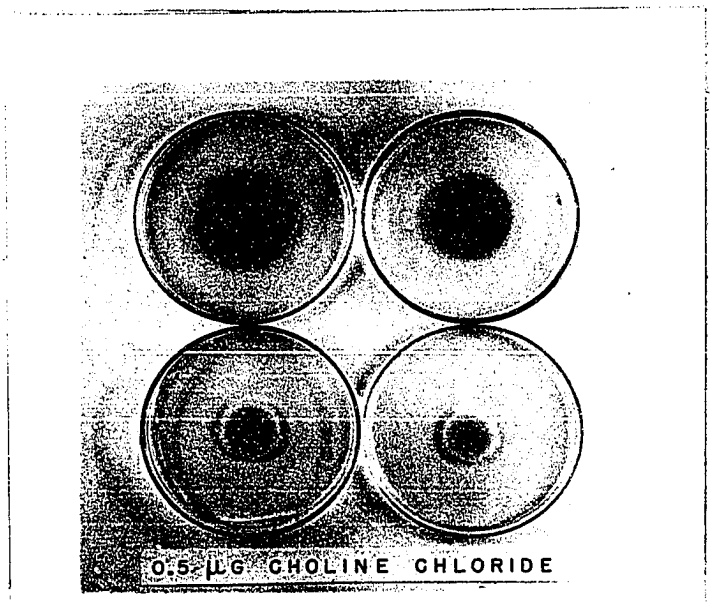


Fig. 15

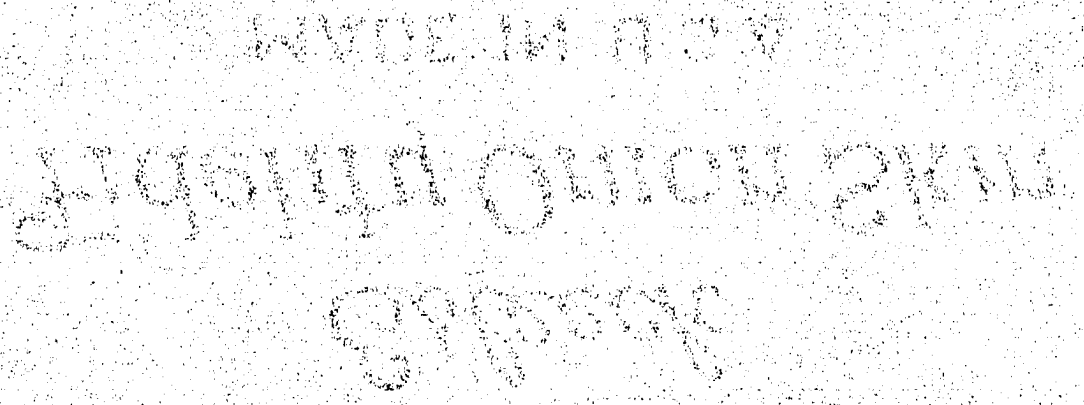
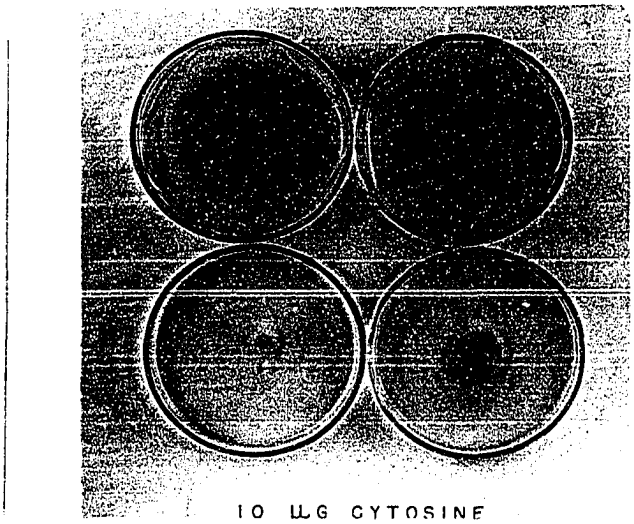


Fig. 16

Growth Changes Caused by the Addition of Cytosine Monohydrate to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



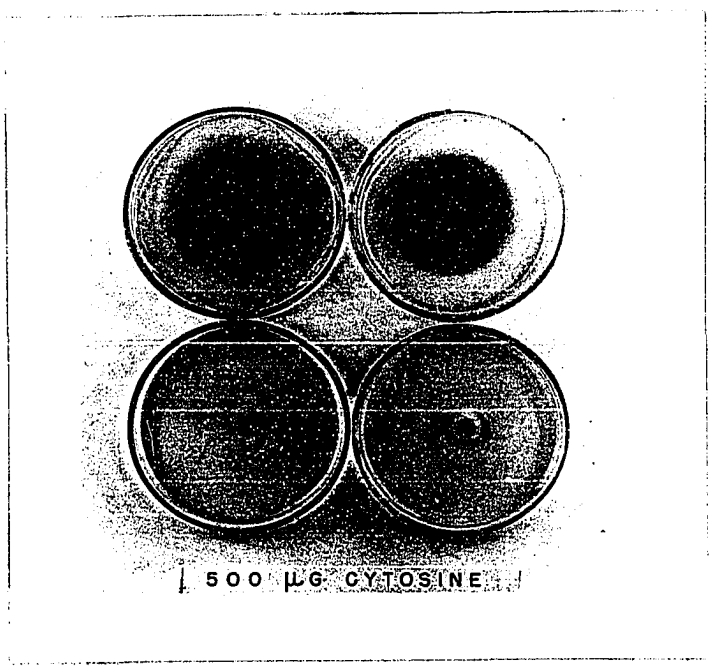
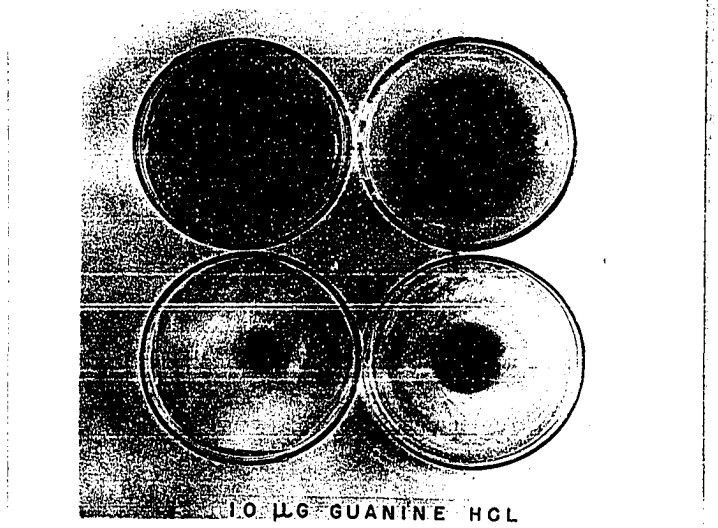


Fig. 16

Fig. 17

Growth Changes Caused by the Addition of Guanine Hydrochloride to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



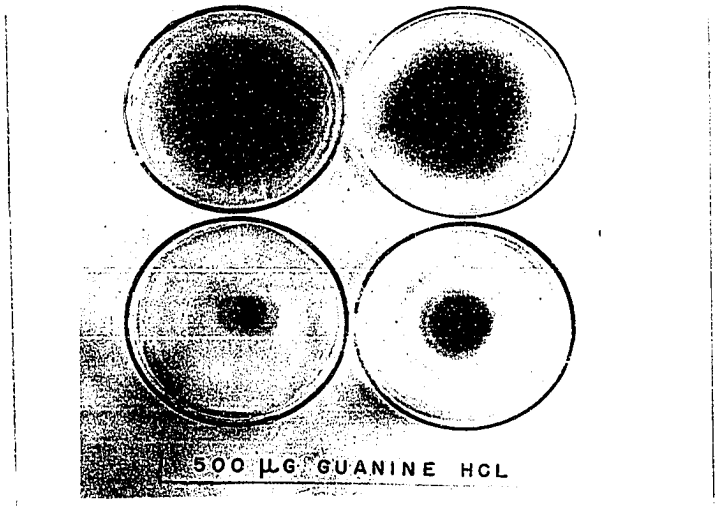
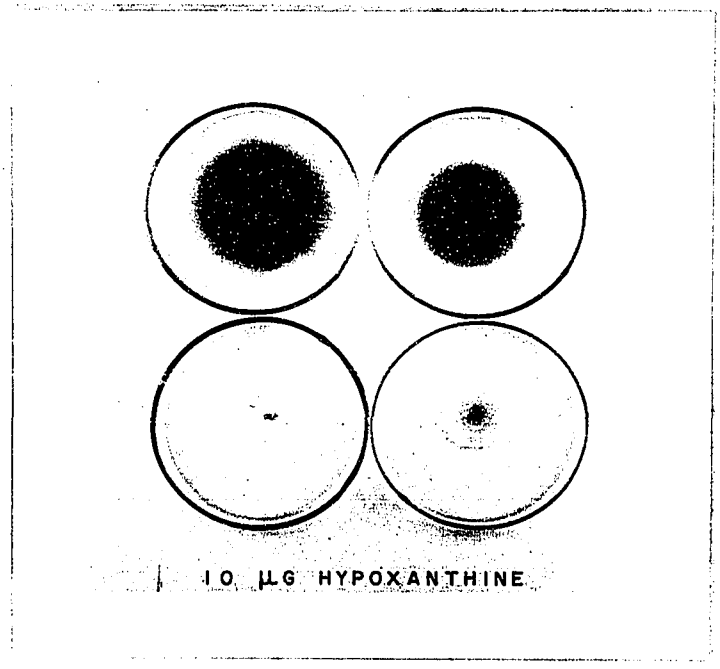


Fig. 17

Fig. 18

Growth Changes Caused by the Addition of Hypoxanthine to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



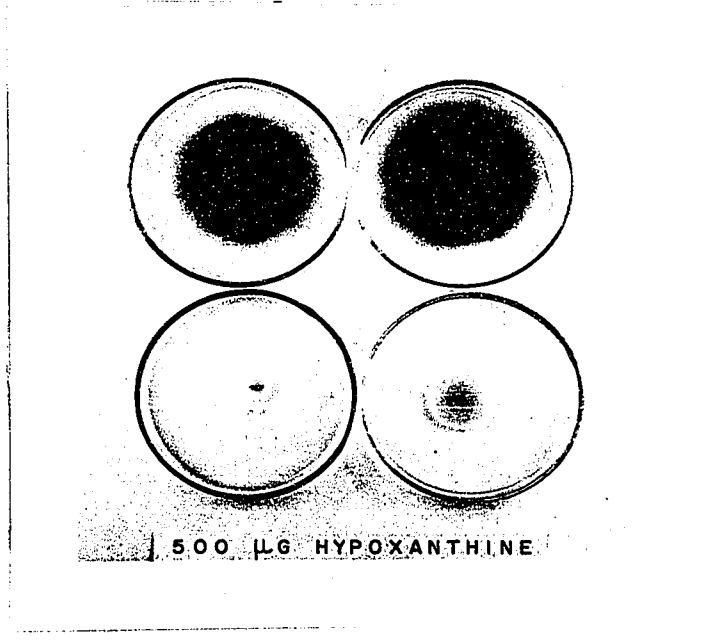


Fig. 18

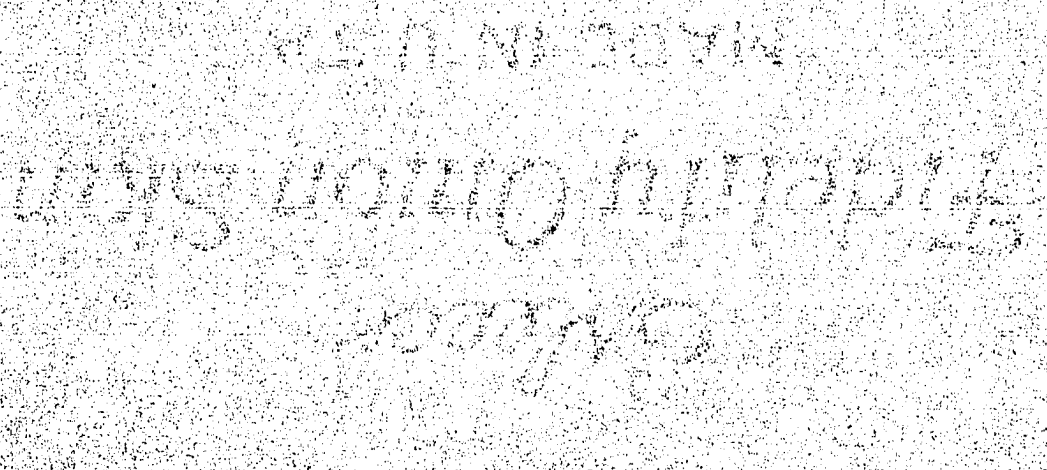
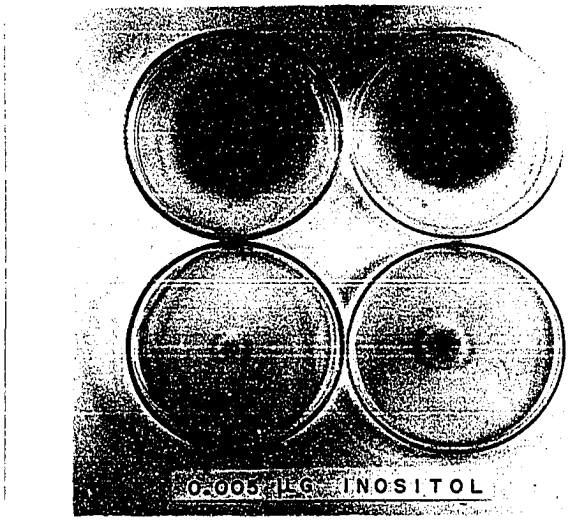


Fig. 19

Growth Changes Caused by the Addition of Inositol to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



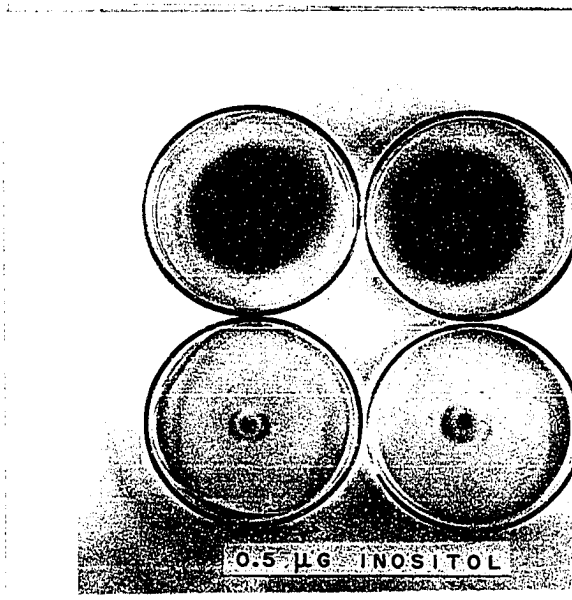
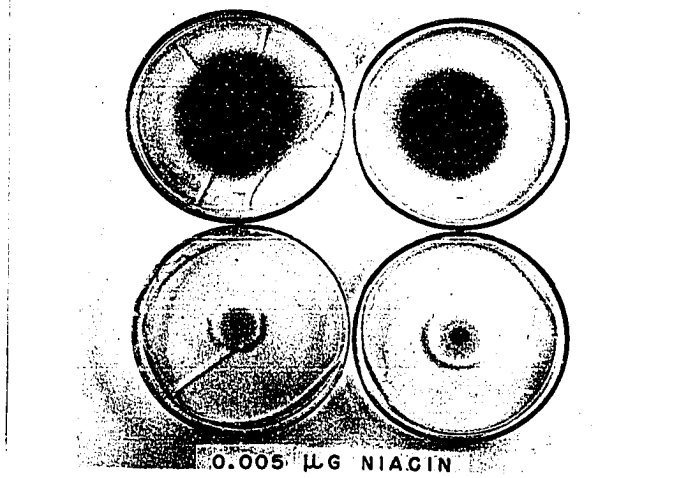


Fig. 19

Fig. 20

Growth Changes Caused by the Addition of Niacin to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



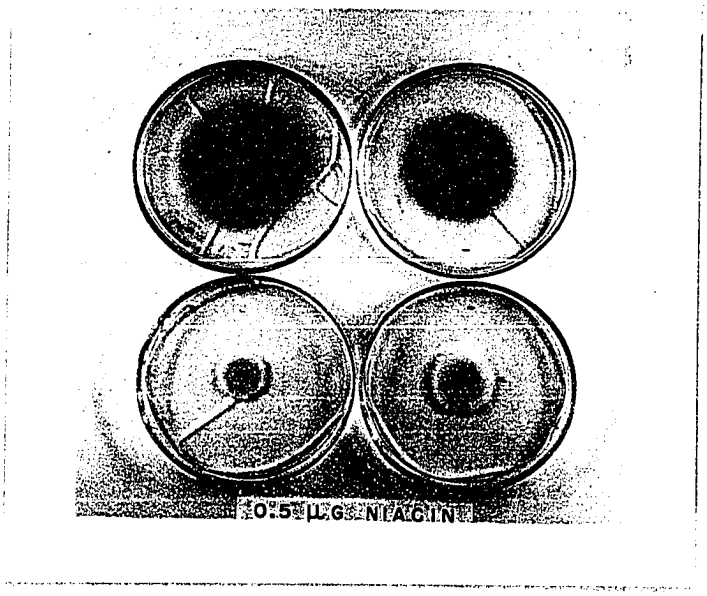
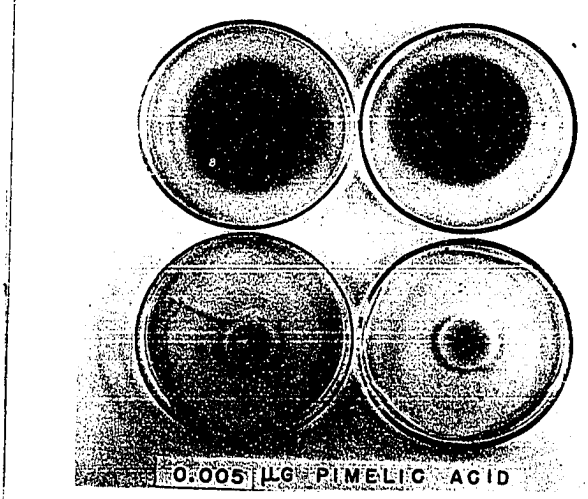


Fig. 20

Fig. 21

Growth Changes Caused by the Addition of Pimelic Acid to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



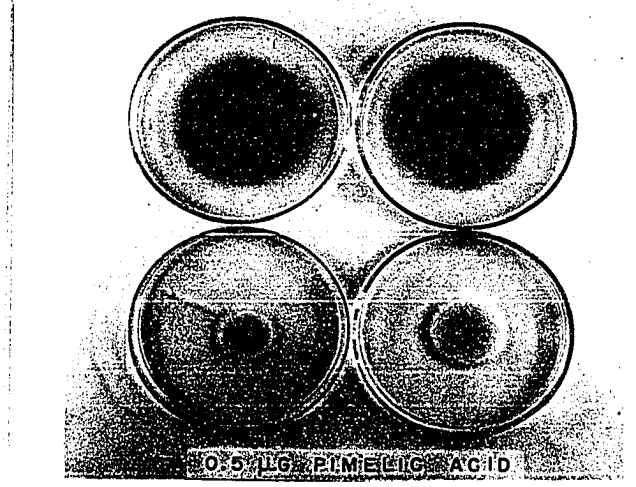
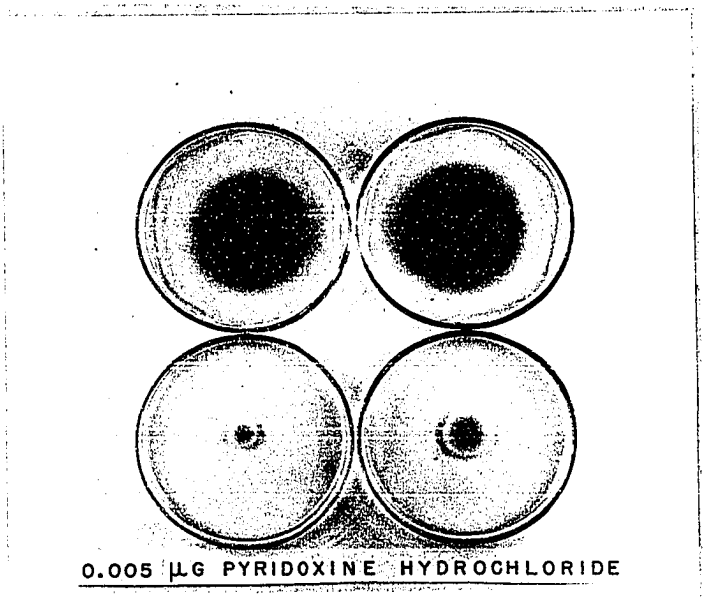


Fig. 21

Fig. 22

Growth Changes Caused by the Addition of Pyridoxine Hydrochloride to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



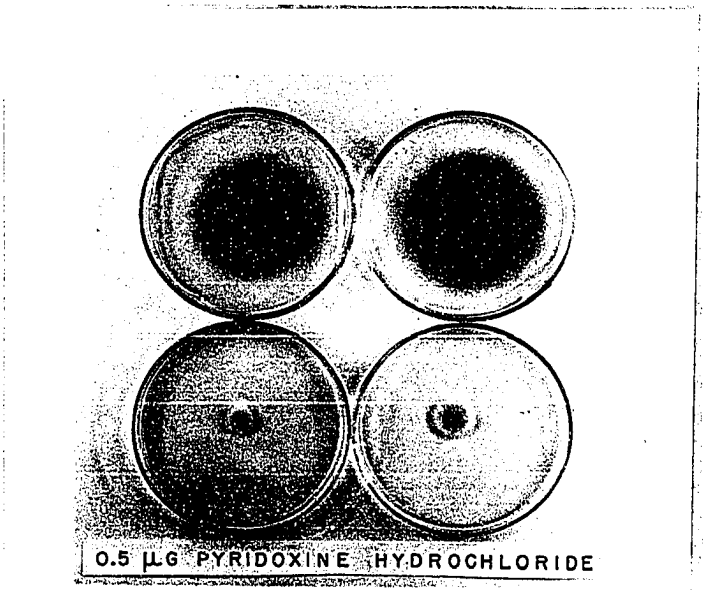


Fig. 22

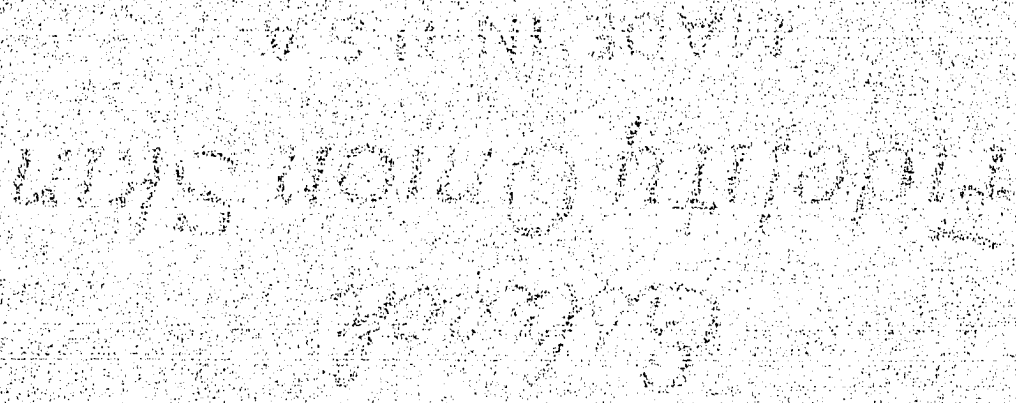
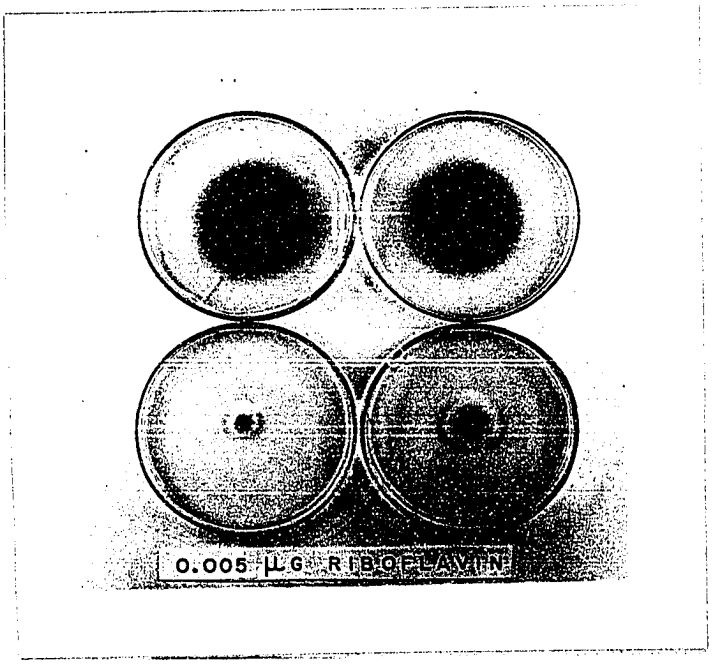


Fig. 23

Growth Changes Caused by the Addition of Riboflavin to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



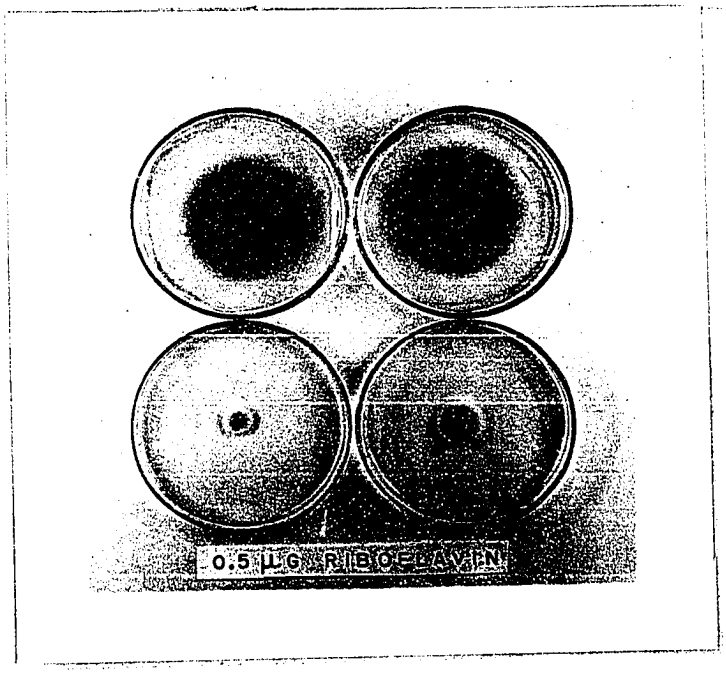
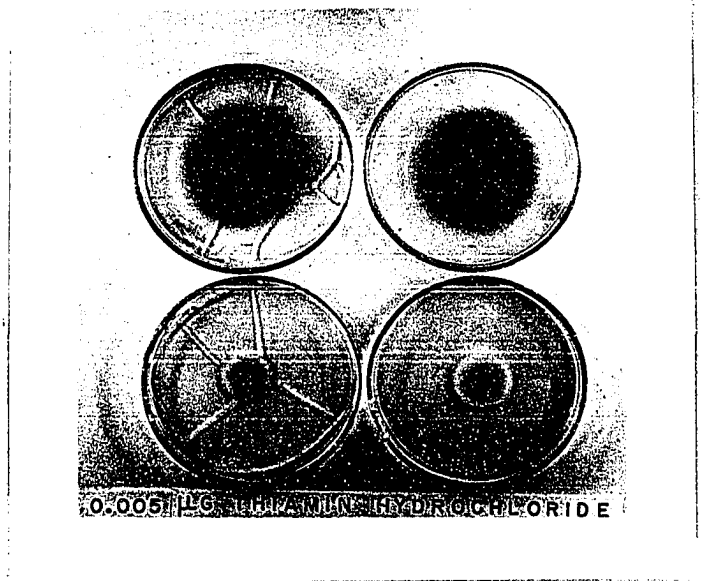


Fig. 23

Fig. 24

Growth Changes Caused by the Addition of Thiamin Hydrochloride to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



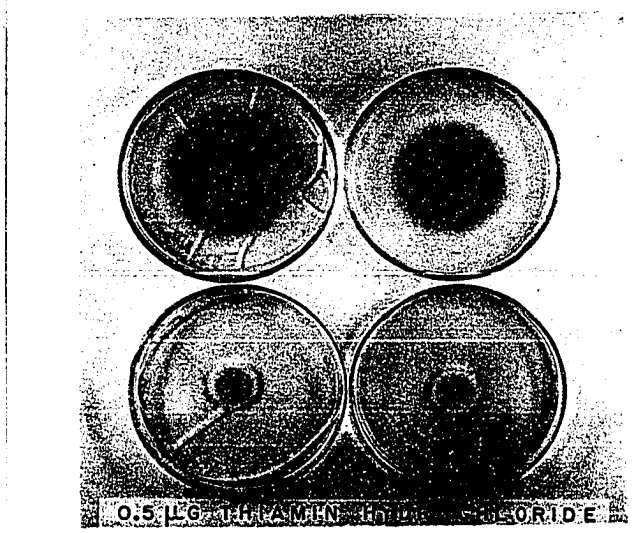
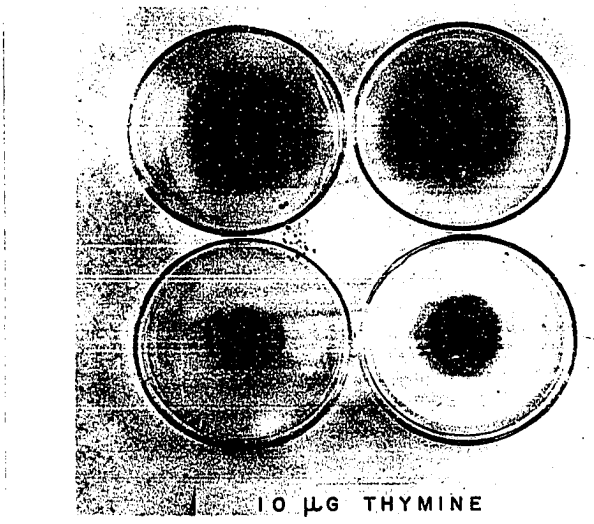


Fig. 24

Fig. 25

Growth Changes Caused by the Addition of Thymine to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



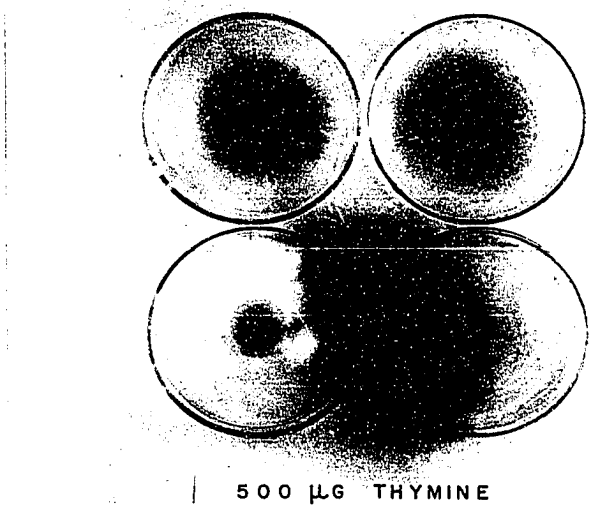
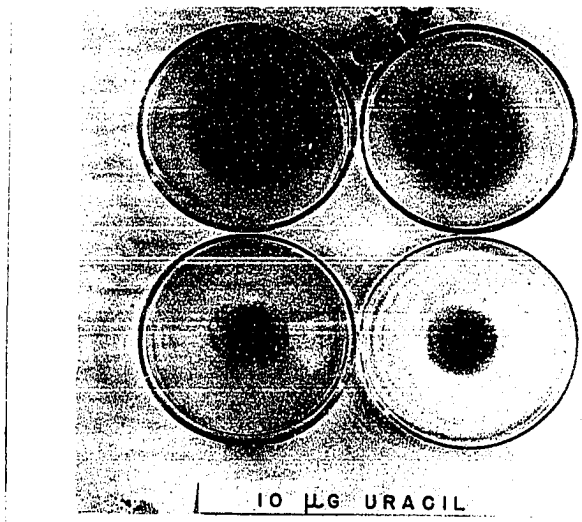


Fig. 25

Fig. 26

Growth Changes Caused by the Addition of Uracil to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



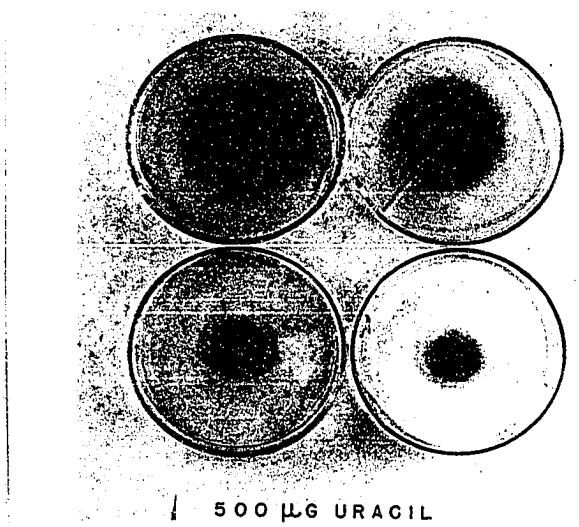
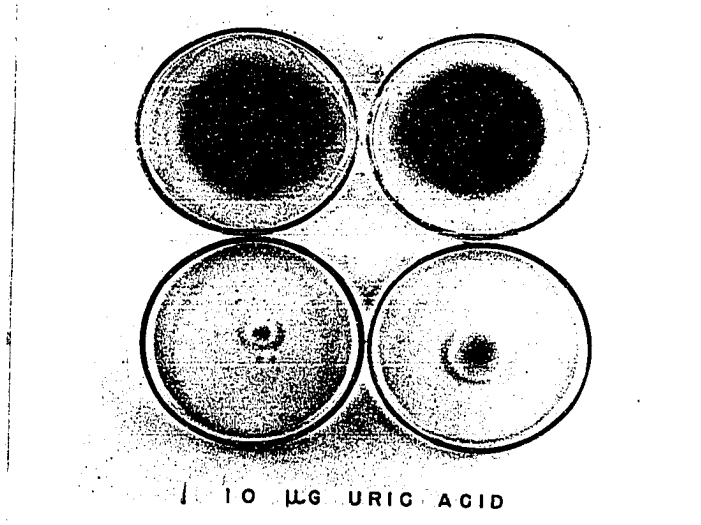


Fig. 26

Fig. 27

Growth Changes Caused by the Addition of Uric Acid to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



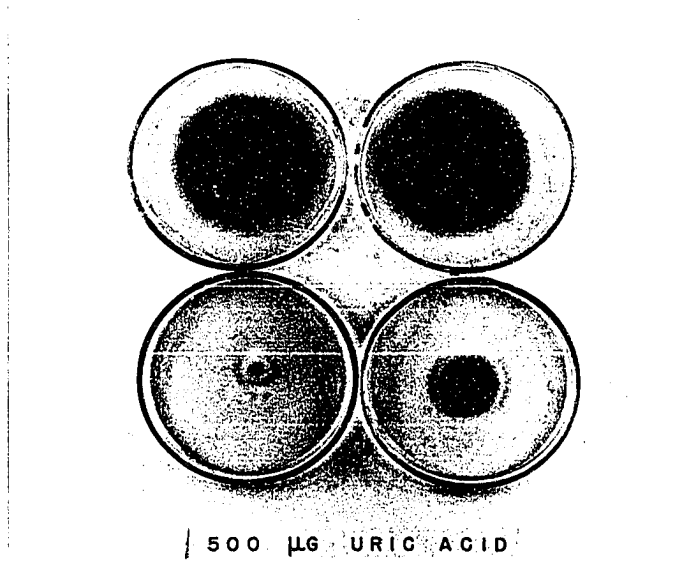
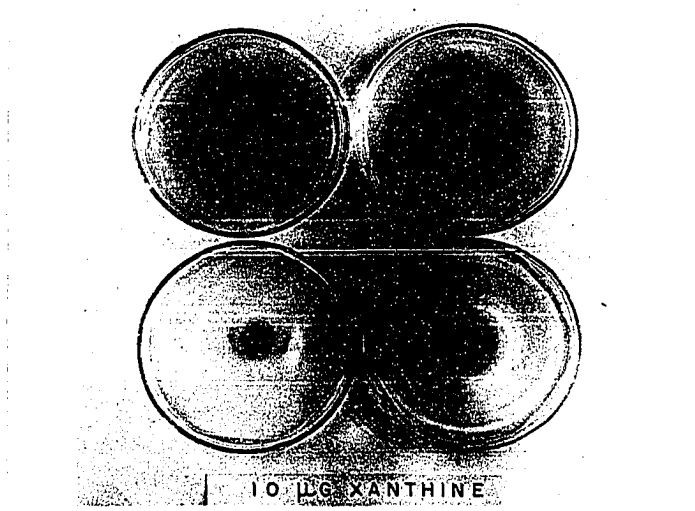


Fig. 27

Fig. 28

Growth Changes Caused by the Addition of Xanthine to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



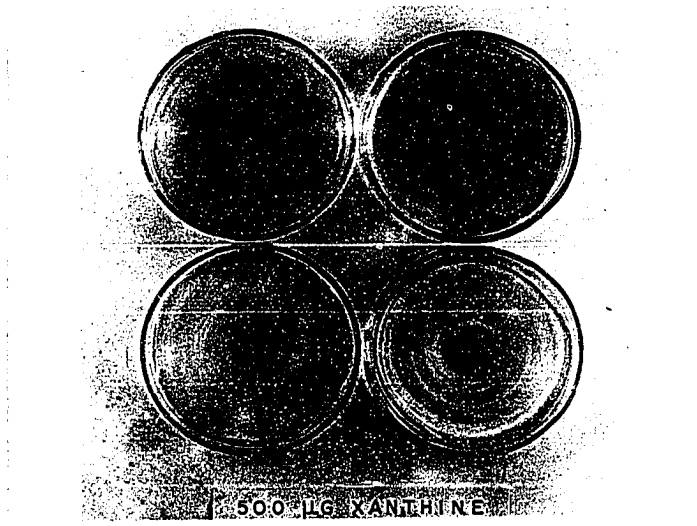
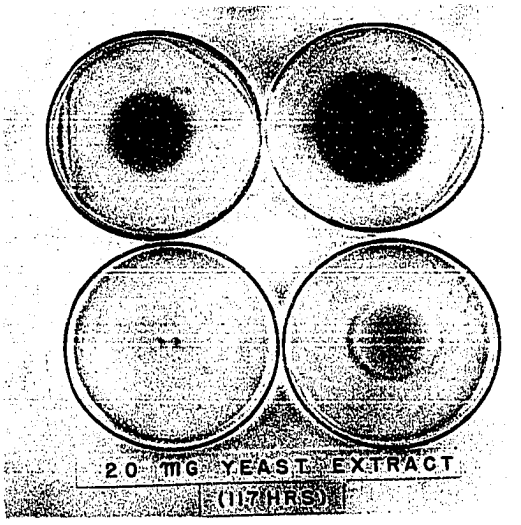


Fig. 28

Fig. 29

Growth Changes Caused by the Addition of Yeast Extract to the Medium

A. niger on basic medium (upper left plate), on medium with growth factor (upper right plate). Mutant on basic medium (lower left plate), on medium with growth factor (lower right plate).



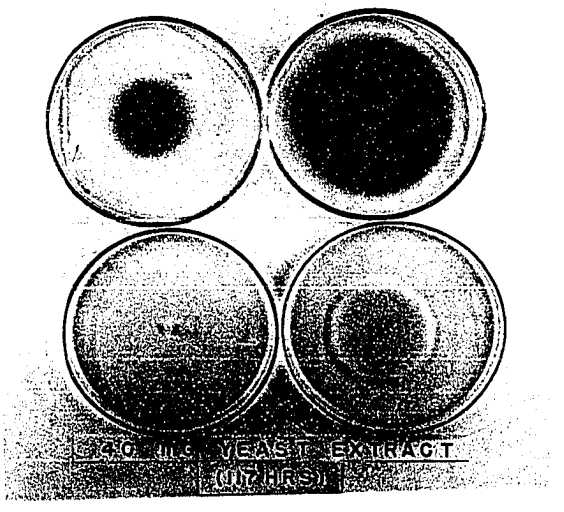


Fig. 29

CHAPTER VI

ANALYSES OF THE NUCLEIC ACID FRACTIONS

Introduction

In an investigation of the differences existing between a mutant and the normal organism, it would seem essential to determine whatever facts one could about the nucleic acid content. The fundamental role of chromatin and chromosomes in heredity is accepted by geneticists.

In the life history of the cell, the part of it designated by biologists as chromatin displays extraordinary activity visible through the microscope. An active arrangement of chromatin structures known as chromosomes usually precedes the process of cell division. (33)

In an attempt to explain the significance of chromosomes in heredity, much work has been done on the chemistry of chromatin with the result that substantial progress has been made in understanding the importance of nucleoprotein material in the composition of chromosomes. Loofbourow has said: "The chromosomes in the nucleus - the rod-shaped bodies in which apparently lie the secrets of the transmission of hereditary characteristics - are almost entirely nucleoproteins." (34)

The work on nucleoproteins has progressed to such a stage that it is possible to connect these substances not only with chromosomes but with genes. "The great accumulation of desoxyribose nucleoproteins in the chromosomes

strongly suggests that these substances are the genes themselves or are intimately related to Genes." (35)

This connection has been elucidated further by Greenstein:

> Study of the fine-structure of certain of the larger chromosomes revealed that the latter are discontinuous, whereby dark-staining bands containing nucleic acid and protein of the histone type alternate with light-staining bands containing protein of the complex type with little or no nucleic acid present. The genic elements are located in the former bands. In this connection it is interesting to note ... that during mitosis the content of histone and desoxypentosenucleic acid run parallel. The gene is thus associated with nucleoprotein of a desoxypentosenucleohistone type; whether it is identified with the latter cannot be definitely answered at the present time.... High efficiencies for mutation of many organisms have been observed in the neighborhood of the wave length of 2600 A at which nucleic acid absorbs very strongly. (36)

It is inevitable that one should agree with the statement that "the basic problem in the study of the genetic effects of irradiation is the physical nature of the changes induced in the genes and chromosomes. This is fundamental not only to problems of induced mutation, but to the larger problems of gene structure and behavior which underlie the whole field of genetics. How does the chromosome in which a mutation has been induced differ, physically and chemically, from an uneffected sister chromosome, and how has this difference been brought about?" (37)

General Procedure

A. niger and mutant tissues, grown on liquid medium, were dried with ethanol and ether. The acid-soluble and the lipoid portions of the dried tissue were removed by extraction. Then the nucleic acid fraction was extracted with 10% NaCl and precipitated from the NaCl solution by lanthanum. Determinations of nitrogen, phosphorus, and pentose (ribose and desoxyribose) were made on the lanthanum precipitable portions of the normal mold and the mutant. In addition the ultraviolet absorption characteristics of the nucleic acid fractions were investigated.

Preparation of Dried Tissue

The medium used for the growth of the tissue was the same as that previously described (pg. 28) except that yeast extract was omitted. The two strains were grown in 500 ml. Ehrlenmeyer flasks each containing 100 ml. of the medium. Incubation was for seven days at 37 degrees.

After the flasks were removed from the incubator, the medium was decanted and discarded. The mold mats were washed several times with large portions of distilled water, and then removed from the flasks onto paper toweling which absorbed surplus moisture. The mats were cut up into small pieces, dried by washing with (1) ethanol, (2) ethanol-ether mixture, (3) ether, and then put into a vacuum desiccator. After a number of days, the material was ground in a mortar,

and returned to the desiccator for the final drying.

Extraction Procedure

The method of extraction was that described by Davidson and Waymouth (38), except for several minor modifications necessitated by the fact that mold tissue rather than animal tissue was being investigated.

In order to remove acid-soluble phosphorus, two grams of the powdered dried mat was weighed out, put into a stoppered 250 ml. centrifuge tube, and shaken for six one-hour periods with successive portions of about 50 ml. 0.1 N HCl. The tissue was then washed with ethanol and extracted for two two-hour periods at 65 degrees under a reflex condenser with successive portions of about 100 ml. of a 3:1 mixture of ethanol and chloroform in order to remove lipoid phosphorus. The material then was dried with ether and put into a vacuum desiccator.

After thorough drying in the desiccator, the nucleic acid extraction was done using 10% sodium chloride solution. 750 mg. of the dried material were put into a 50 ml. conical centrifuge tube, 2 drops of triacetin added, and extraction carried out with five successive portions of 10% NaCl under the following conditions: (a) 15 ml. at 0° for 4 hours, (b) 5 ml. at 0° overnight, (c) 4 ml. at 100° for 30 minutes, (d) 3 ml. at 100° for 10 minutes, (e) 3 ml. at 100° for 10 minutes. The five NaCl extracts were com-

bined and made up to 30 ml.

At three different times, extractions were made with HCl, ethanol-chloroform, and NaCl as described above. A fourth extraction was made in which trichloroacetic acid was substituted for the HCl. The use of trichloroacetic acid was suggested by Mann's work (39) on the phosphorus content of A. niger v. Tiegh. The extraction was carried out with six portions of trichloroacetic acid: (a) 25 ml. of 10% acid at 0° for one hour, (b) four 25 ml. portions of 5% acid at 0° for one hour each, and (c) 25 ml. of 5% acid at 0° overnight.

Lanthanum Precipitation

Nine ml. of the NaCl extract were pipetted into a 50 ml. conical centrifuge tube; 1 ml. of 2.5% lanthanum nitrate and 10 ml. of ethanol were added. The tube was kept at 0° for one hour and then centrifuged. The supernatent was discarded and the precipitate washed twice with 3 ml. of 0.25% lanthanum nitrate. The precipitate was decomposed with 1 ml. of 0.5 M Na_2CO_3 and 8 ml. of water added. After centrifuging, the precipitate (lanthanum carbonate) was discarded and the supernatent, containing the lanthanum precipitable portion, was used for the various phosphorus, nitrogen, pentose, and absorption tests.

Phosphorus Determination

Phosphorus determinations were made on the lanthanum precipitable portions of the sodium chloride extracts according to the method of Fiske and SubbaRow (40). Total phosphorus was determined on all four of the extractions and inorganic phosphorus on the second extraction (Table 10).

Nitrogen Determination

Nitrogen determinations were made on the lanthanum precipitable portions of the four extractions of A. niger and the mutant by the colorimetric micro-method which employs Nessler's reagent (41). Calculations of nucleic acid content were made, assuming nucleic acid equals six times the nitrogen concentration. Results are shown in Table 11.

Ultraviolet Absorption

Various ultraviolet absorption curves were obtained using a Beckman quartz spectrophotometer. The amount of absorption of ultraviolet light by the solutions is reported as optical density.

$$O.\ D. = 2 - \log_{10} G$$

where G is the per cent transmission of light by the solution.

$$G = 100(I/I_o)$$

where I_o is the intensity of the incident beam of light and I is the intensity of the beam emerging from the solution.

The region investigated was that around 260 millimicrons, so the wavelength was varied from 235 to 300 millimicrons.

The following data were collected:

(1) variation in optical density with wavelength for yeast nucleic acid (12.5 μg./ml.) and sodium desoxyribonucleate (20 μg./ml.), given in Table 12 and Figure 30;

(2) variation in optical density for various concentrations of ribonucleic acid (from yeast) from 5 μg./ml. to 40 μg./ml with wavelength fixed at 260 mμ, given in Table 13 and Figure 31;

(3) variation in optical density with wavelength for the lanthanum precipitable portions of ribonucleic acid, sodium desoxyribonucleate, and bile salt extract of Clostridium perfringens, given in Table 14 and Figure 32;

(4) variation in optical density with wavelength for the lanthanum precipitable portions of the four sodium chloride extracts obtained from the normal A. niger and from the mutant, given in Tables 15 and 16 and Figures 33 and 34;

(5) total nucleic acid concentrations of the lanthanum precipitable portions of the four sodium chloride extracts of A. niger and of the mutant, based on the standard ribonucleic acid curve, given in Table 17.

Table 10. Phosphorus Determinations on Lanthanum Precipitable Portions of *A. niger* and Mutant

Extraction	Total Phosphorus	
	A. niger (μg./ml.)	Mutant (μg./ml.)
First	78	43
Second	85	36
Third	85	37
Fourth	67	39

Extraction	Inorganic Phosphorus	
	A. niger (μg./ml.)	Mutant (μg./ml.)
Second	< 2	< 2

Table 11. Nitrogen Determinations and Nucleic Acid Concentrations (Based on Nitrogen) for the Lanthanum Precipitable Portions of *A. niger* and Mutant

Extraction	*A. niger*		Mutant	
	Nitrogen	Nucleic acid (6 x N)	Nitrogen	Nucleic acid (6 x N)
	µg./ml.	µg./ml.	µg./ml.	µg./ml.
First	29	174	27	162
Second	31	186	26	156
Third	29	174	26	156
Fourth	51	306	40	240

Table 12. Ultraviolet Absorption Data - Standard Solutions of Ribonucleic Acid (Yeast) and Sodium Desoxyribonucleate (Thymus)

Wavelength (mμ.)	RNA (12.5 μg./ml.) O.D.	DRNA (20 μg./ml.) O.D.
235	- - -	0.198
240	0.058	0.233
250	0.243	0.358
260	0.284	0.431
270	0.238	0.376
280	0.155	0.252
290	0.054	0.108
300	0.027	0.024

Fig. 30

Variation of Optical Density with Wavelength for Standard Solutions of Ribonucleic Acid (Yeast) and Sodium Desoxyribonucleater (Thymus)

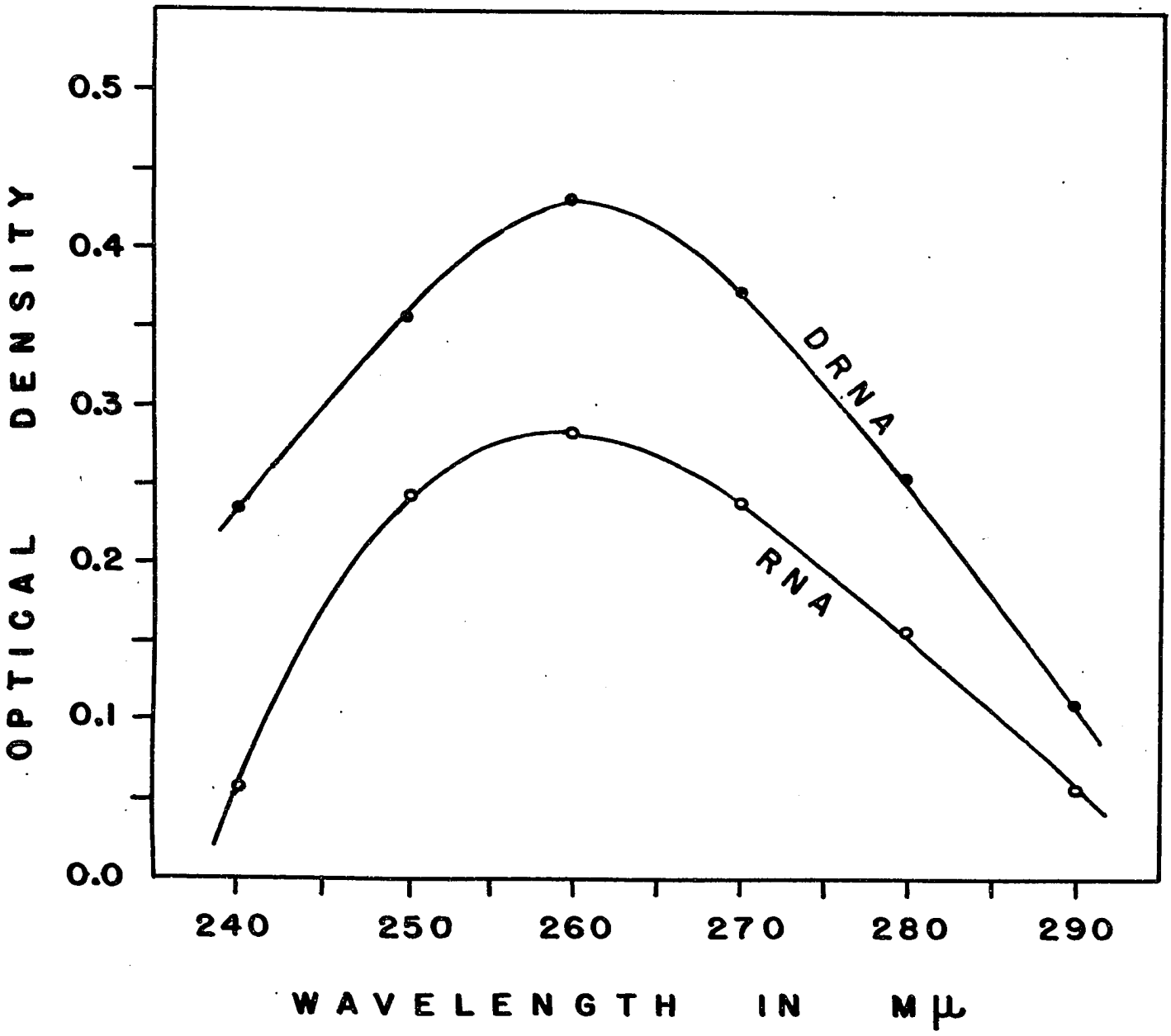


Table 13. Ultraviolet Absorption Data - Various Concentrations of Ribonucleic Acid (Yeast) at 260 mμ.

Concentration RNA (μg./ml.)	Optical Density
5	0.111
10	0.257
15	0.376
20	0.493
25	0.625
30	0.778
35	0.900
40	1.030

Fig. 31

Variation of Optical Density with Concentration for Solutions of Ribonucleic Acid (Yeast) at 260 mμ.

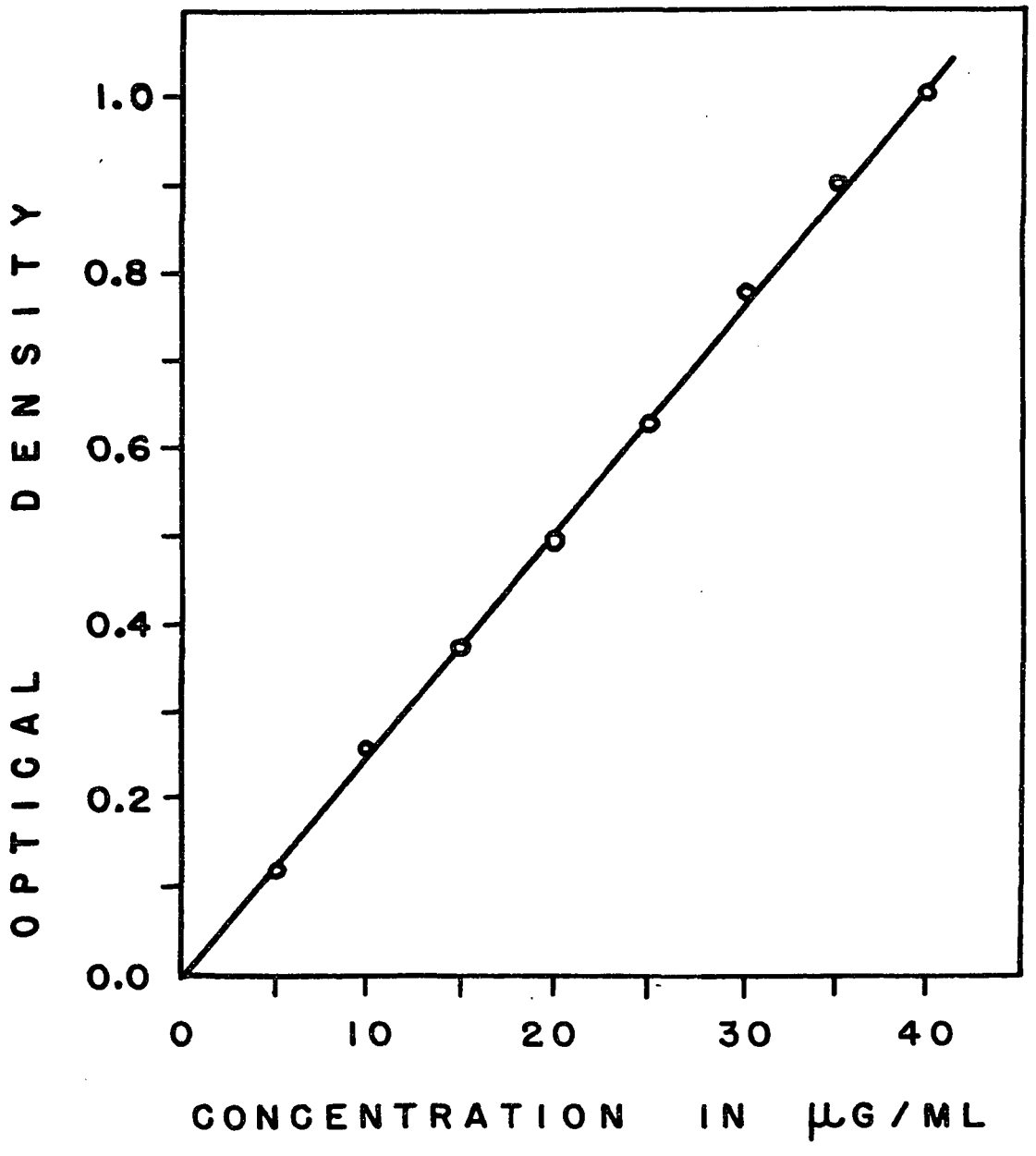


Table 14. Ultraviolet Absorption Data for the Lanthanum Precipitable Portions of Ribonucleic Acid (Yeast), Sodium Desoxyribonucleate (Thymus), and Bile Salt Extract of Clostridium perfringens

Wavelength (mμ.)	Na desoxyribonucleate O.D.	Ribonucleic acid O.D.	Bile Salt Extract of Clostridium perfringens (1:200 dilution) O.D.
235	0.405	0.340	0.250
240	0.425	0.425	0.293
250	0.628	0.608	0.384
260	0.685	0.655	0.400
270	0.598	0.525	0.308
280	0.402	0.317	0.187
290	0.185	0.128	0.076
300	0.062	0.026	0.022

Fig. 32

Variation of Optical Density with Wavelength for the Lanthanum Precipitable Portions of Ribonucleic Acid (Yeast), Sodium Desoxyribonucleate (Thymus) and Bile Salt Extract of *Clostridium perfringens*.

Curve A, for ribonucleic acid; B, for sodium desoxyribonucleate; C, for *Clostridium perfringens*

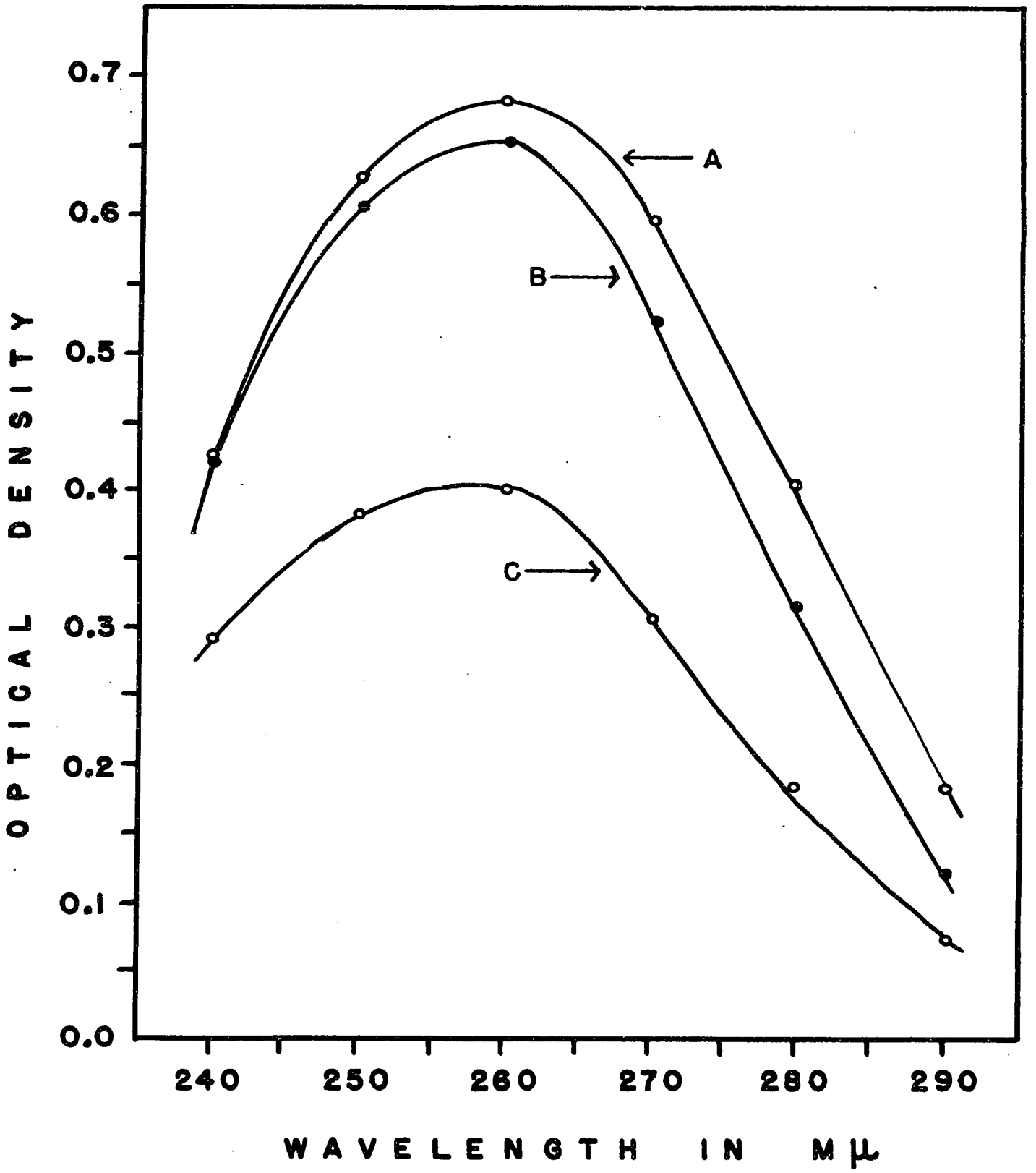


Table 15. Ultraviolet absorption Data - Lanthanum Precipitable Portions of the Sodium Chloride Extracts of *A. niger*

Wave-length (mμ.)	First Extraction (diluted 1:20) O. D.	Second Extraction (diluted 1:20) O. D.	Third Extraction (diluted 1:10) O. D.	Fourth Extraction (diluted 1:10) O. D.
235	- - -	- - -	0.355	0.525
240	0.081	0.068	0.338	0.570
250	0.204	0.208	0.412	0.742
260	0.233	0.235	0.440	0.785
270	0.201	0.201	0.365	0.648
280	0.131	0.132	0.231	0.388
290	0.065	0.059	0.103	0.148
300	0.032	0.027	0.038	0.032

Fig. 33

Variation of Optical Density with Wavelength for the Lanthanum Precipitable Portions of Four Sodium Chloride Extracts of *A. niger*.

Curve A, from first extraction; B, from second extraction; C, from third extraction; D, from fourth extraction

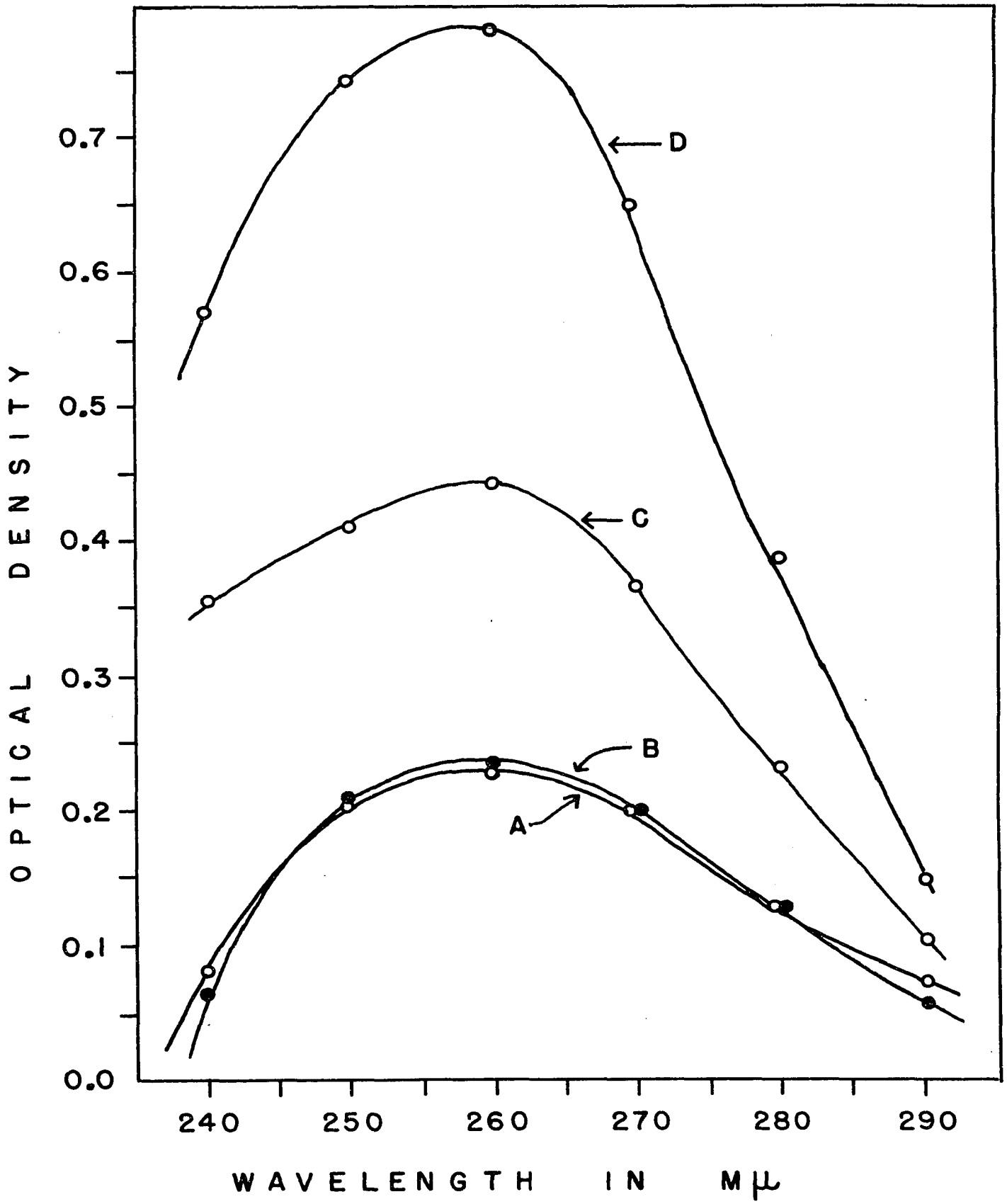


Table 16. Ultraviolet Absorption Data - Lanthanum Precipitable Portions of the Sodium Chloride Extracts of Mutant *A. niger*

Wavelength (mμ.)	First Extraction (diluted 1:10) O. D.	Second Extraction (diluted 1:10) O. D.	Third Extraction (diluted 1:10) O. D.	Fourth Extraction (diluted 1:10 O. D.
235	- - -	- - -	0.334	0.443
240	0.201	0.157	0.325	0.480
250	0.360	0.370	0.387	0.585
260	0.399	0.386	0.412	0.608
270	0.347	0.376	0.350	0.485
280	0.225	0.271	0.225	0.307
290	0.104	0.161	0.100	0.128
300	0.046	0.092	0.043	0.042

Fig. 34

Variation of Optical Density with Wavelength for the Lanthanum Precipitable Portions of Four Sodium Chloride Extracts of Mutant *A. niger*

Curve A, from first extraction; B, from second extraction C, from third extraction; D, from fourth extraction

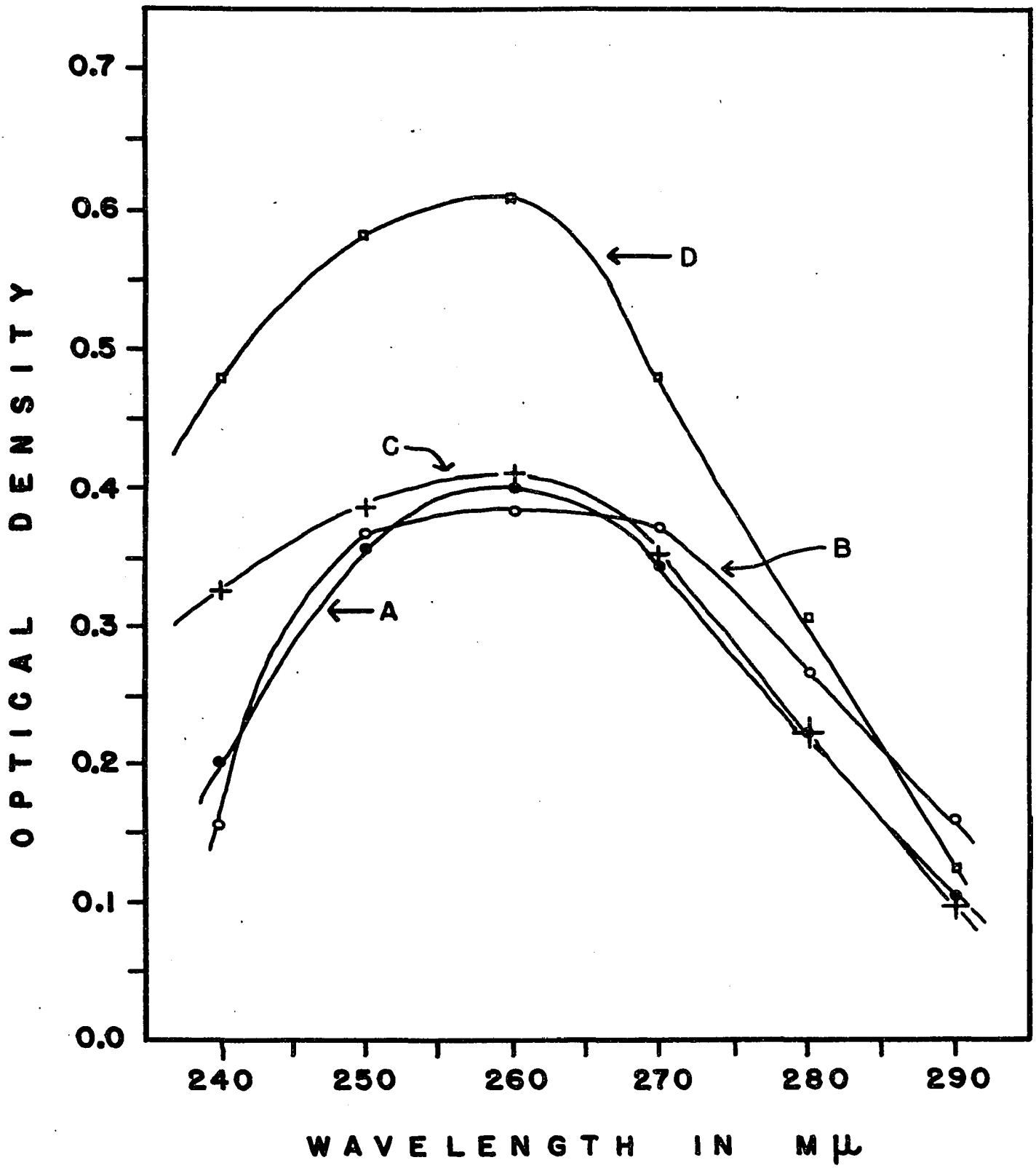


Table 17. Total Nucleic Acid Concentrations of Lanthanum Precipitable Portions of A. niger and Mutant Based on the Standard Ribonucleic Acid Curve, Fig. 31, using Data for Absorption at 260 mμ. from Tables 15 and 16

Extraction	O. D.	Concentration of NA in diluted sample (μg./ml.)	Dilution	Concentration of NA in lanthanum precipitate (μg./ml.)
		A. niger		
First	0.233	8.8	1:20	176
Second	0.235	9.0	1:20	180
Third	0.440	17.7	1:10	177
Fourth	0.785	30.7	1:10	307
		Mutant		
First	0.399	15.5	1:10	155
Second	0.386	15.0	1:10	150
Third	0.412	16.2	1:10	162
Fourth	0.608	23.8	1:10	238

Ribose Determination

Ribose was determined by two methods: (a) that employing the aniline acetate reagent and (b) the perchloric acid-tryptophane method. The determination by the first method was done by a modification of the procedure used by Davidson and Waymouth (38), based on the method of Reeves and Munro (42). It is known that with this method one can expect recovery of no more than 50% of the ribose content of the sample. Two ml. of the lanthanum precipitable portion were pipetted into a 25 x 200 ml. test tube to which 2 ml. of 5.55 N HCl and 5 ml. of xylene were added. The tube was fitted to a reflux condenser and submerged in a boiling water-bath for 2.5 hours. After cooling, the xylene layer was removed, using a small separatory funnel, and 2 ml. of xylene were added to this layer. Then 4 ml. were removed to a dry test tube and 5 ml. of freshly made aniline acetate reagent added. The color was allowed to develope in a dark room for 20 minutes. Concentration was determined by comparing the unknown solutions in the spectrophotometer at 520 mμ. against a standard curve (Table 18 and Figure 35) developed using furfural solutions to which the aniline acetate reagent had been added.

The standard furfural solutions had been made by dissolving furfural in xylene, assuming 1 ml. furfural equals 1159 mg. furfural, and 23.18 μg. furfural are comparable to

45.40 µg. ribose.

The results of this ribose test on the nucleic acid fractions from the first extraction of the normal A. niger and the mutant are shown in Table 19.

Determination of ribose using the perchloric acid-tryptophane reaction was done according to the procedure given by Cohen (43). Two ml. of the lanthanum precipitate being investigated, 0.5 ml. of 1 per cent dl-tryptophane, 2.5 ml. of 60 per cent perchloric acid constituted the reaction mixture which was subjected to the temperature of an autoclave at 15 lbs. pressure for one hour. After cooling, the color was extracted with 5 ml. of isoamyl alcohol, and absorption measured in the spectrophotometer at 640 mµ. Concentration was determined by comparison with a standard curve (Table 20 and Figure 36) for absorption of ribose solutions subjected to the test. Results, including calculation of ribonucleic acid content based on ribose, are given in Table 21.

Table 18. Ribose Determination (Aniline Acetate Reagent) - Absorption Data at 520 mμ. for Standard Amounts of Ribose Based on Furfural

Furfural (μg.)	Ribose (μg.)	O. D.
2.32	4.54	0.027
4.63	9.08	0.116
6.95	13.62	0.197
9.27	18.16	0.288
11.59	22.70	0.399
13.91	27.24	0.471

Fig. 35

Ribose Determination (Aniline Acetate Reagent) - Variation of Optical Density with Concentration for Standard Solutions of Ribose (Based on Furfural), at 520 mμ.

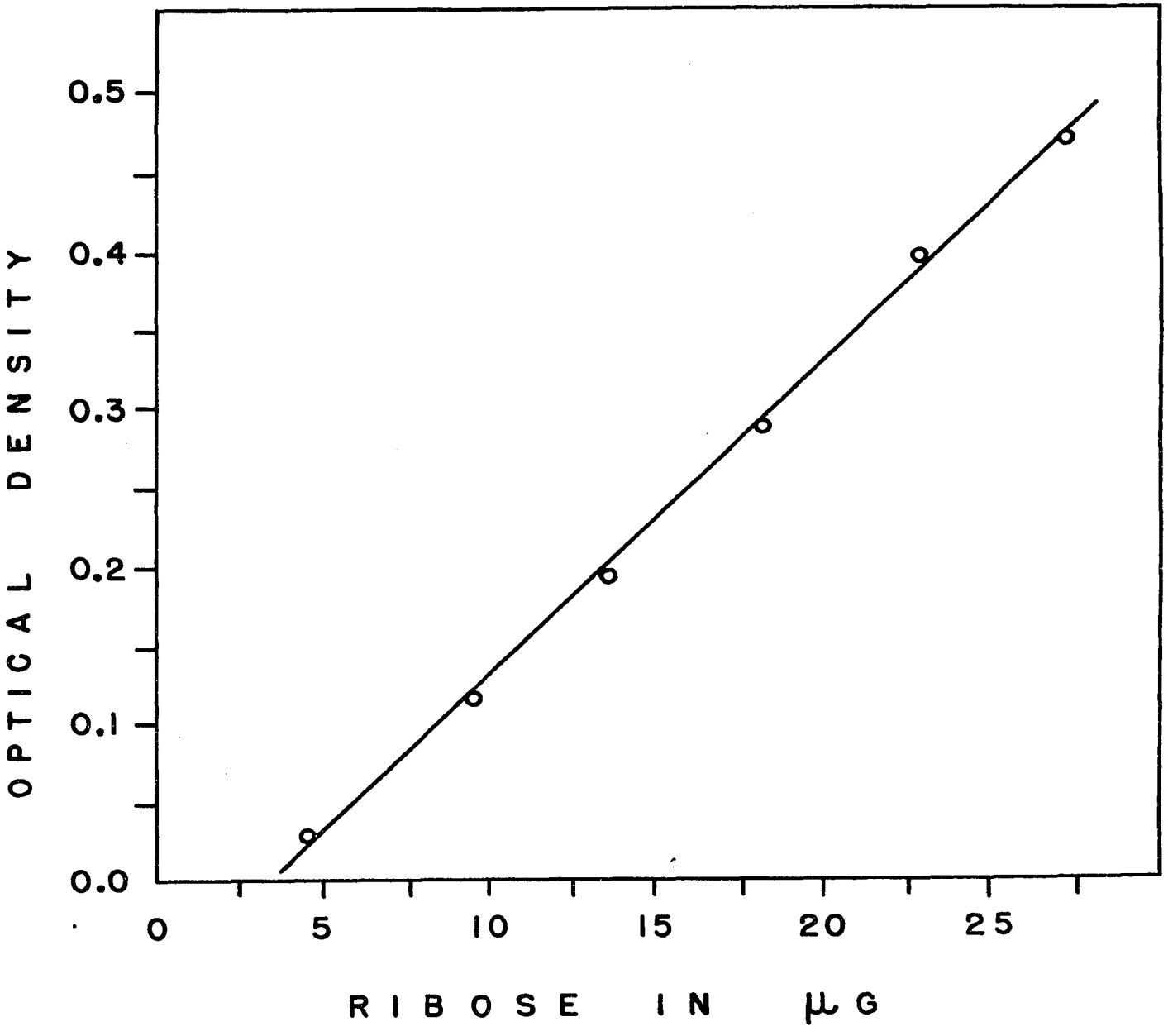


Table 19. Ribose Determination (Aniline Acetate Reagent) - Absorption Data at 520 mμ. for Lanthanum Precipitable Portions (First Extraction) of A. niger and Mutant

Organism	Ml. Used	O. D.	Ribose Concentration from Standard Curve (μg./ml.)
A. niger	2	0.184	11.1
Mutant	2	0.132	9.0

Table 20. Ribose Determination (Perchloric Acid-Tryptophane Method) - Absorption Data at 640 mμ. for Standard Amounts of Ribose

Ribose	O. D.
25	0.243
50	0.405
75	0.560
100	0.693

Fig. 36

Ribose Determination (Perchloric Acid-Tryptophane Method) - Variation of Optical Density with Concentration for Standard Amounts of Ribose at 640 mμ.

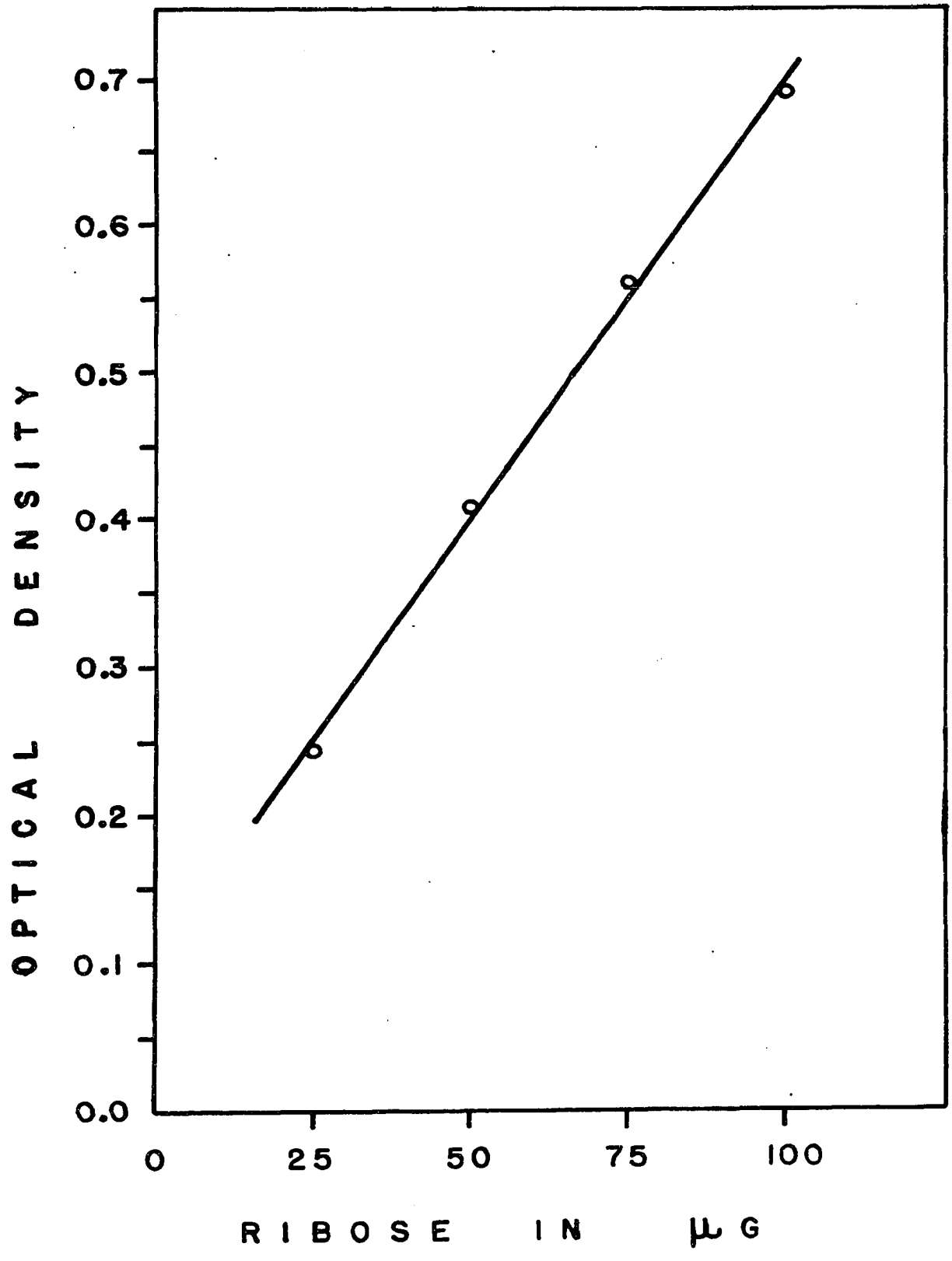


Table 21. Ribose Determination (Perchloric Acid-Tryptophane Method) - Absorption Data at 640 mμ. for Lanthanum Precipitable Portions of A. niger and Mutant

Extraction	Ml. Used	O. D.	Ribose Concentration from Standard Curve (μg./ml.)	RNA Concentration (RNA=3.2 ribose) (μg./ml.)
		A. niger		
First	2	0.548	38	122
Third (First sample)	2	0.530	36	115
(Second sample)	2	0.542	37	118
		Mutant		
First	2	0.492	33	106
Third (First sample)	2	0.500	34	109
(Second sample)	2	0.486	32	102

Desoxyribose Determination

Determinations of desoxyribose were made using (a) the diphenylamine reagent and (b) the perchloric acid-tryptophane method. The use of the diphenylamine reagent was carried out according to the procedure followed by Davidson and Waymouth (38). It is known that with this method one can expect recovery of no more than 50% of the desoxyribose content of the sample. Absorption was measured at 600 mμ. with the spectrophotometer and concentration calculated by comparison with a standard curve (Table 22 and Figure 37) obtained using solutions of sodium desoxyribonucleate. Results are given in Table 23.

Determination of desoxyribose by the perchloric acid-tryptophane method was performed according to the procedure of Cohen (43). Two ml. of the lanthanum precipitate being investigated, 0.4 ml. of 1% dl-tryptophane, and 2.4 ml. of 60% perchloric acid constituted the reaction mixture which was heated in a boiling water bath for 10 minutes. After cooling, the color was extracted with 3 ml. of isoamyl alcohol and absorption determined with the spectrophotometer at 500 mμ. Calculation of desoxyribonucleic acid content was made by comparison with a standard curve (Table 24 and Figure 38) drawn for various amounts of sodium desoxyribonucleate subjected to the same test. The results of these calculations are shown in Table 25.

Table 22. Desoxyribose Determination (Diphenylamine Reagent) - Absorption Data at 600 mμ. for Standard Amounts of Sodium Desoxyribonucleate

DRNA (μg.)	O. D.
40	0.009
60	0.020
80	0.033
150	0.106
200	0.148
250	0.187

Fig. 37

Desoxyribose Determination (Diphenylamine Reagent) - Variation of Optical Density with Concentration for Standard Amounts of Sodium Desoxyribonucleate at 600 mμ.

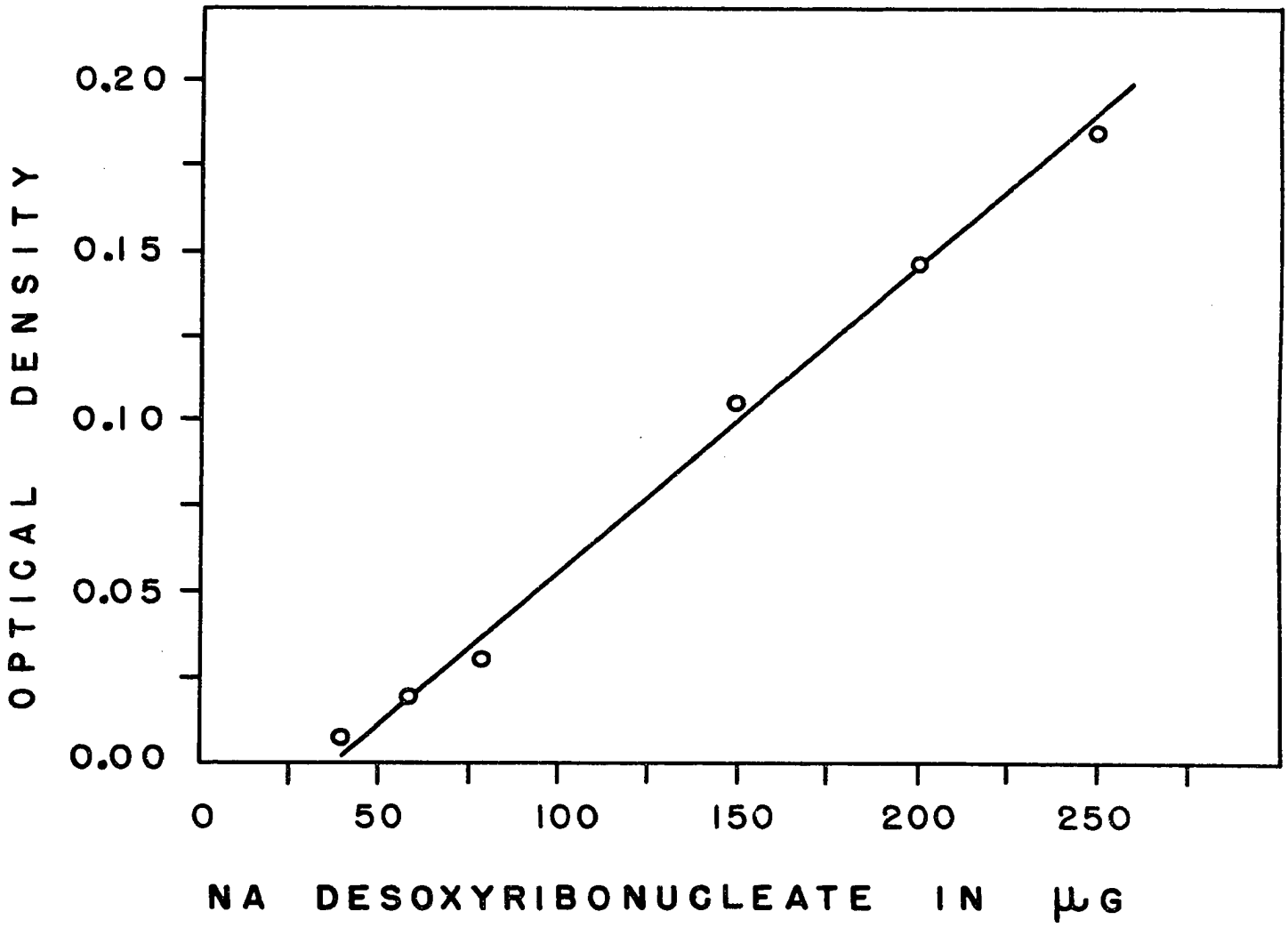


Table 23. Desoxyribose Determination (Diphenylamine Reagent) - Absorption Data at 600 mμ. for Lanthanum Precipitable Portions (Second Extraction) of A. niger and Mutant

Organism	Ml. Used	O. D.	DRNA Concentration Based on Standard Curve (μg./ml.)
A. niger	3	0.020	27
Mutant	3	0.014	24

Table 24. Desoxyribose Determination (Perchloric Acid-Tryptophane Method) - Absorption Data at 500 mμ. for Standard Amounts of Sodium Desoxyribonucleate

DRNA (μg.)	O. D.
25	0.014
50	0.033
75	0.050
100	0.070
150	0.109

Fig. 38

Desoxyribose Determination (Perchloric Acid-Tryptophane Method) - Variation of Optical Density with Concentration for Standard Amounts of Sodium Desoxyribonucleate at 500 mμ.

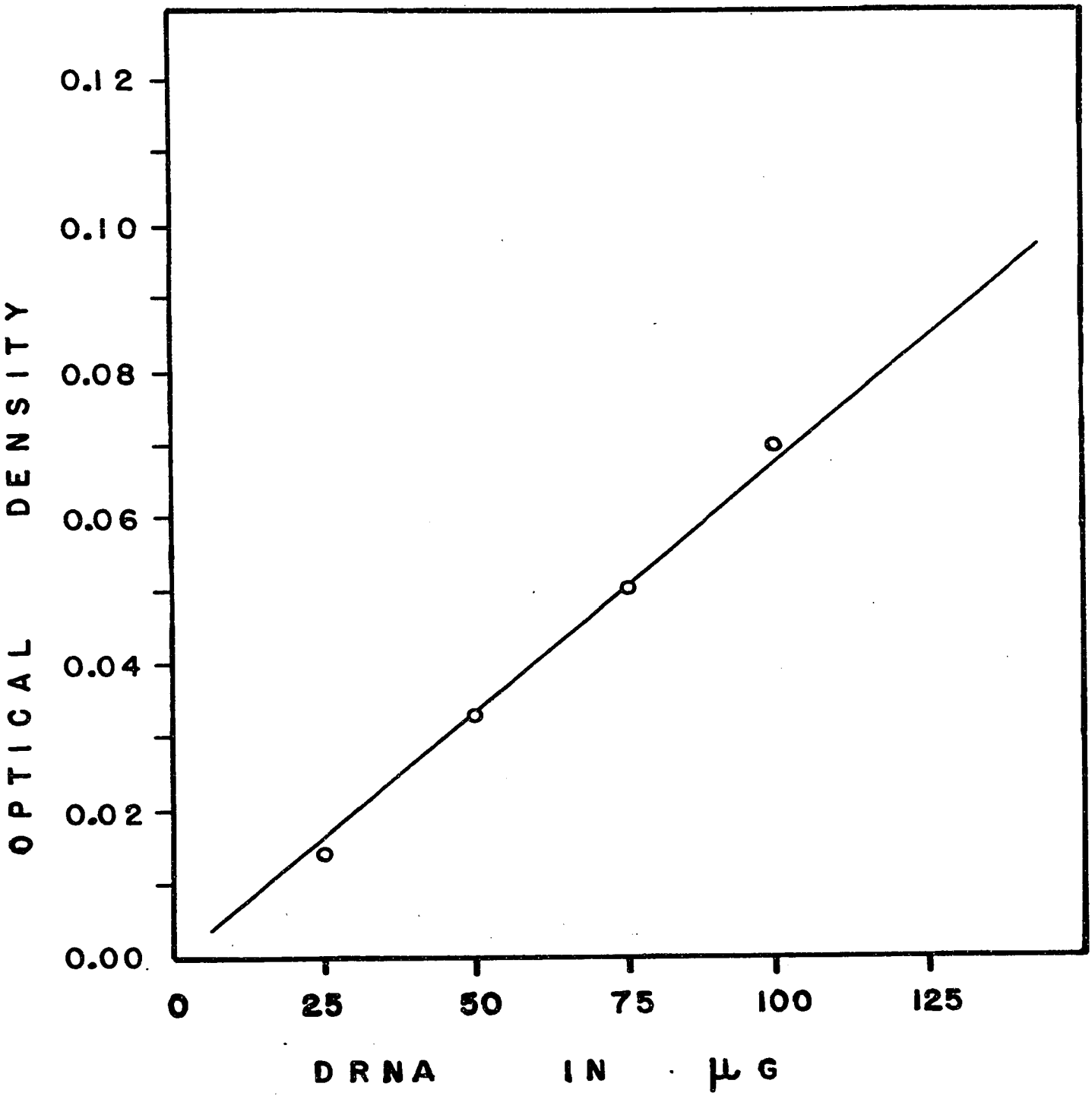


Table 25. Desoxyribose Determination (Perchloric Acid-Tryptophane Method) - Absorption Data at 500 mμ. for Lanthanum Precipitable Portions of *A. niger* and Mutant

Extraction	Ml. Used	O. D.	DRNA Concentration from Standard Curve (μg./ml.)
		A. niger	
First	2	0.063	47
Third (First sample)	2	0.064	48
(Second sample)	2	0.069	52
Fourth	2	0.083	61
		Mutant	
First	2	0.052	39
Third (First sample)	2	0.057	42
(Second sample)	2	0.053	40
Fourth	2	0.092	68

Summary

The comparison of the nucleic acid content of the lanthanum precipitable portions of the four sodium chloride extracts of A. niger and the mutant is given in Table 26. Because of the low recovery known to be present with the use of the aniline-acetate and diphenylamine reagents, NA concentrations based on these results are not given.

Using the average values for the first three extractions, a comparison of the two strains with respect to concentration of phosphorus, nitrogen, total nucleic acid, ribose, and desoxyribonucleic acid is given in Table 27.

Because of the distorted phosphorus-nitrogen ratio obtained for the first three extractions, the fourth extraction was done according to the method suggested by Mann (39) but this did not yield appreciably different phosphorus values; in addition, the nitrogen concentrations were higher than the previous ones. In order to clarify the situation the absorption spectra in the ultraviolet region were investigated.

Table 26. Comparison of Nucleic Acid Content of A. niger and Mutant, Based on Three Different Methods

Extraction	NA based on nitrogen (µg./ml.)	NA based on Absorption at 260 mµ. (µg./ml.)	DRNA + RNA based on perchloric acid-tryptophane method (µg./ml.)
		A. niger	
First	173	176	169
Second	184	180	167
Third	172	177	-
Fourth	306	307	-
		Mutant	
First	160	155	145
Second	155	150	147
Third	154	162	-
Fourth	239	238	-

Table 27. Comparison of the Analyses of the Lanthanum Precipitable Portions (Nucleic Acid Fractions) of A. niger and Mutant

Substance	Method	Ratio of Concentrations, A. niger / Mutant
Phosphorus	Fiske-SubbaRow	2.13 / 1
Nitrogen	Micro-Kjeldahl (Colorimetric)	1.15 / 1
Nucleic Acid	Absorption at 260 mμ.	1.14 / 1
Ribose	Aniline-acetate reagent	1.23 / 1
Ribose	Perchloric acid-tryptophane	1.12 / 1
Desoxyribonucleic acid	Diphenylamine reagent	1.13 / 1
Desoxyribonucleic acid	Perchloric acid-tryptophane	1.23 / 1

The unique qualities of the absorption spectrum of nucleic acid are discussed by Loofbourow (34). This author has shown experimentally that the ultraviolet absorption spectrum of thymus nucleic acid is equivalent to the sum of the absorption spectra of its purine and pyrimidine constituents. He also reviews the general characteristics of the absorption spectra of a number of substances of biochemical interest in order to show how they differ from one another. These characteristics may be summarized as follows:

carbohydrates

most sugars - rapid rise from about 2300 A. to shorter wavelengths

fructose - maximum at 2800 A.

sorbose - maximum at 2750 A.

fatty acids

saturated - rapid rise from 2500 A. to shorter wavelengths

unsaturated - single broad absorption band

amino acids

tyrosine - in acid solution, maximum 2750-2800 A.

- in alkaline solution, maximum 2850-2900 A.

tryptophane - maximum, 2750-2800 A.

phenylalanine - maximum, 2560-2575 A.

others not containing

an aromatic nucleus - rise from 2500-2200 A. to shorter wavelengths

purines and pyrimidines

adenine - maximum at 2625 A.

guanine - maxima at 2460 and 2730 A.

uracil - maximum at 2600 A.

thymine - maximum at 2625 A.

cytosine - maximum at 2650 A.

nucleic acids

broad maximum at 2600 A.

minimum at 2300 A.

In order to test the validity of the characteristics of the absorption spectrum of nucleic acid, investigation was made of the spectra of material isolated from various sources and prepared by various methods. Absorption curves were obtained for

(a) RNA (Schwartz) purified by reprecipitation from acetic acid according to the method of Kunitz (44), given in Figure 30,

(b) DRNA prepared as the sodium salt from thymus by the methods of Mirsky and Pollister (45) and McCarty (46), given in Figure 30,

(c) the lanthanum precipitable portions of RNA and DRNA prepared by the method of Davidson and Waymouth (38), given in Figure 32,

(d) the lanthanum precipitable portion of the bile salt extract of Clostridium perfringens which had been prepared by a modification of the method of Henry and Stacey (47), given in Figure 32,

(e) the lanthanum precipitable portions of the three sodium chloride extracts of A. niger and mutant prepared by the method of Davidson and Waymouth (38), given in Figures 33 and 34,

(f) the lanthanum precipitable portions of the sodium chloride extracts of A. niger and mutant prepared by a combination of the methods of Mann (39) and Davidson and Waymouth (38), given in Figures 33 and 34.

All these curves showed the maximum absorption at 2600 Angstroms which is typical of nucleic acids. On the basis of the absorption at 2600 A., it was found that (a) the fourth extraction showed higher nucleic acid concentration than the first three extractions for both the normal mold and the mutant, (b) the NA concentrations for the first three extractions agreed among themselves, and (c) the NA concentrations for the first three extractions agreed with those calculated from the nitrogen content.

CHAPTER VII

CONCLUSIONS

In none of the observations or determinations did the normal A. niger and the mutant strains show the same characteristics. The differences in the two strains may be summarized as follows:

1. The mutant is cream to light tan in color instead of very dark brown, sporulation is depressed, and growth is slower than for the normal A. niger.

2. The mutant is more efficient in the production of citric acid, producing more citric acid with a utilization of less glucose.

3. The mutant is more responsive to individual growth substances than the normal A. niger, indicating loss of synthesizing ability on the part of the mutant.

4. All tests on the lanthanum precipitable portions of the two strains indicate a lesser nucleic acid content for the mutant.

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