FACTORS AFFECTING THE VARIABILITY OF A CHOLESTEROL-UTILIZING STREPTOMYCETE

A Thesis

Presented to

The Faculty of the Department of Biology

The University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Microbiology

by

Salvador Joseph Sedita

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ABSTRACT

This thesis is an account of various factors which influence the observed variability of a cholesterol utilizing Streptomyces species.

Preliminary studies were carried out to determine the feasibility of using mycelial fragments of the test organism as plating units and as inoculum. Two mutants were isolated using nitrosoquanidine as mutagenic agent.

Genetic recombination between the two auxotrophs was demonstrated and confirmed. Further, it was shown that genetic recombination could be a factor in the variability of the test organism Streptomyces I_4 pH_8 #2 (w).

Storage of spores and their subsequent use as inoculum or as plating units was shown to play a role in variability. Loss of the ability to utilize cholesterol on storage makes it advisable to use careful check procedures to assure the presence of this or any particular physiological trait.

Growth on other carbon sources than cholesterol was demonstrated to affect the subsequent ability of the organism to utilize cholesterol.

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I. Introduction

Lewis (1962) presented evidence that certain species of the genus Streptomyces are capable of using cholesterol as the sole source of carbon. This report constituted the first evidence for the use of cholesterol by this genus (Peterson and Davis, 1964; Davis et al., 1963; Lewis, 1962). Two isolates were characterized in detail, Streptomyces I_3 pH_4 and Streptomyces I_4 pH_8. The latter is a "Halo" former while the former produces no clearing, or "Halo", around the colony on solidified cholesterol medium. Strain I_4 pH_8 also produced a variant which upon primary isolation produced a "Halo" but which subsequently lost this ability (Davis, 1962).

Initial work with these organisms was impeded by the instability of morphological and physiological characteristics (Lewis, 1962). The problem was partially overcome by selection of proper media for maintenance and propagation, but no satisfactory method for the long term storage with preservation of physiological characteristics has been achieved. These observations are not at all unexpected as the actinomycetes and especially the genus Streptomyces have long been considered by many investigators to be extremely variable. Such variability makes it difficult to use streptomycetes in industrial processes and makes difficult the interpretations of laboratory investigations.

A study of techniques was undertaken to determine the feasibility of using spores and mycelial fragments as plating units for investigation. Since spores are normally used as plating units, comparisons were made with mycelial fragments obtained from growth on media containing 0.5% agar and from liquid cultures. The primary purpose of such a study was to provide a satisfactory basis for the use of mycelial fragments when cultural conditions are such that the organism does not produce spores.

Further studies were undertaken in an attempt to provide satisfactory storage conditions for the maintenance of the organism as laboratory stocks with stable physiological and morphological characteristics. A decrease in the number or per cent of spores or fragments capable of utilizing cholesterol was used to determine the effect of various other carbon sources on the ability to utilize cholesterol.

Experiments were designed to demonstrate genetic recombination between auxotrophic mutants of the organism. These experiments might also demonstrate that genetic phenomena are involved in the physiological variability observed.

Long term storage and continuous subculture appeared to be major factors in the loss of the cholesterol utilizing character. Culture in the presence of some carbon source, other than choles-

terol, could successfully regenerate this lost characteristic (Davis, 1962) and was used as the basis for studies to determine the effect of other carbon sources on the organism's cholesterol utilizing capability.

II. Literature Review

a.) Development of Techniques

A major problem in the study of the genetic interactions of the streptomycetes is the difficulty of obtaining material suitable for analysis. Ideally, such material should be available in large quantity and possess the characteristics of a uniform suspension. Obtaining suitable amounts of material is not a problem; however, unlike the single-celled bacteria, mycelium from cultures of Streptomyces spp. cannot be dispersed uniformly in a liquid medium. The delivery of equal samples of these organisms, within the experimental framework, can therefore be only crudely approximated.

In the past genetic analysis of Streptomyces spp. has commonly been carried out using suspensions of spores harvested from the surface of a suitable sporulation medium. Moyer, et al. (1937) first used the technique of homogenizing mycelium. In an effort to obtain uniform inoculum suspensions for the gluconic acid process, submerged or surface mycelium from Aspergillus niger strains was macerated, or homogenized, in a Waring blender. Since growth starts from each viable particle regardless of size, then the greater the number of viable particles in a given volume, the more rapid the development time and the shorter the overall time for the whole process (Foster, 1949). Savage and

Vander Brook (1946) demonstrated in the penicillin process, that the homogenized mycelium of Penicillium species diluted as much as 40,000 times, served as an adequate substitute for unblended submerged mycelium used at a 1:10 seeding rate. Blending greatly increases the efficiency of mycelium used as inoculum and allows for ease of handling by pipetting (Foster, 1949).

Although high speed blenders have been employed in the fragmentation of mycelium (Savage and Vander Brook, 1946; Dorrell and Page, 1947; Moore and Mason, 1951), early blenders were too large for the preparation of small quantities of material. Friedhoff and Rosenthall (1954) reported that the procurement of suitable equipment and the control of contamination present difficulties when blenders are used. Availability of micro-scale equipment is no longer a problem. Contamination still presents minor difficulties, due mainly to leakage through the gaskets around the cutting blades of most commercial blenders and to the large size of the opening of the containers. Hand grinding of mycelium in a mortar and pestle is unsatisfactory, mainly due to the risk of contamination and to the non-uniform size of the resultant fragments.

In the fungi the multicellular structure of the mycelium creates problems in the measurement of growth of the filamentous forms. Surface area is a poor index of growth since the rate of

growth of species with differing colonial characteristics cannot be compared with any degree of accuracy. The same difficulties which occur using fungal mycelia apply to the streptomycetes and the other objections, regarding surface area as an index of growth, are well taken. Suspensions prepared from the mycelium of streptomycetes should represent a more nearly uniform suspension than similar suspensions of fungi, since the mycelium itself more nearly approaches the diameter of the true bacteria than does the mycelium of filamentous fungi.

Although spore suspensions, derived from the growth of Streptomyces on a solid medium, have been used extensively in the past, objections have been raised to this procedure. Most of the objections have been to the use of fungal spore suspensions, but should apply to any spore suspension. Dorrell and Page (1947) have pointed out the existence of a latent period in spores as an obvious disadvantage. In experimental infections, spores do not simulate the parasitic phase of dermatophytes in the same manner as do mycelia, as has been pointed out by Raubitschek (1955). Another obvious disadvantage is that experimental work is necessarily limited to sporogenous strains of fungi. This applies as well to the streptomycetes and one of the most common of the variations observed is the loss of spore formation.

The use of spore suspensions, while very convenient, has other implications; for example, Okami et al. (1963) demonstrated agar to influence the morphology and pigmentation of streptomycetes. Additionally, Snider (1963) pointed out that the analysis of spore suspensions is not completely satisfactory because it represents only an indirect study of the conditions which may prevail in the mycelium. Gregory and Huang (1964a, 1964b) have shown that control cultures of mycelial fragments of Streptomyces scabies give rise to 10 times more tyrosinase deficient colonies than do their spores.

In an analysis of nuclear ratios in Schizophyllum commune, Snider (1963) used mycelial fragments, as the organism produces no asexual spores. In principle the technique is similar to the classical methods utilizing spore suspensions, but, as pointed out by Snider, the use of mycelial fragments is particularly adapted to the analysis of very disparate ratios and is probably suitable for the simultaneous comparison of ratios in mycelium and spores.

Snider's analysis was made using a Waring blender to homogenize the mycelium. Gregory and Huang (1964a, 1964b) also used this technique with Streptomyces scabies. Guidry and Trelles (1962), however, used a technique which seems to be considerably more effective and applicable to smaller amounts of material.

These authors used a Duall tissue grinder and their technique allows for the preparation of a more uniform suspension of mycelial fragments by allowing for carefully controlled maceration of the test material.

b.) Investigations of Variability

The variability of actinomycetes has been the subject of a great deal of study in recent years. Environmental influences as factors in the variations observed in Streptomyces spp. have been studied by many workers including Lieske, 1921 (see Jones, 1954), Waksman, 1922; Jones, 1940; Duggar et al., 1954; Wilkins and Rhodes, 1955; Erikson, 1955; Carvajal, 1953; Welsch, 1959; Bradley, 1958; and Von Plotho, 1940.

Jones (1940) demonstrated the usefulness of colonial characteristics in the taxonomy of Streptomyces spp. when synthetic media and standard cultural conditions are maintained. This concept, previously presented by Krainsky (1914) and Waksman (1919), has been more thoroughly studied (Burkholder et al., 1954; Hesseltine et al., 1954) and appears to have gained acceptance.

Some of the most common types of variation can be described as a loss of spore formation, changes from smooth to rough colonies, rise of spore-forming variants, pigment loss, lethal variations, and changes in the biochemical properties of the organisms.

Jones (1954) classified the variations observed in the streptomycetes on the basis of temporary variations and permanent variations. The former result directly from environmental

effects such as the amount or age of inoculum, effect of medium, etc. Permanent variations cause changes in temperature requirements, odor, spores, etc. These appear to be increased by factors well known as mutagenic agents, e.g., X-rays, ultraviolet radiation, nitrous acid, etc. Phage can also be considered a factor which affects permanent variations (Carvajal, 1953; Shirling, 1953; and Bradley, 1957).

Genetic mechanisms as factors in the variability of streptomycetes have achieved prominence in recent years. Heterokaryosis, originally observed in filamentous fungi (see Pontecorvo, 1946), was mentioned briefly by Lederberg (1955) and later by Sermonti and Spada-Sermonti (1955) in investigations with Streptomyces coelicolor. Bradley and Lederberg (1956) and Szybalski and Braendle (1956) presented more extensive evidence for heterokaryosis in this group of organisms.

The investigations of some of these workers indicated that recombination was being observed (Sermonti and Spada-Sermonti, 1956; Szybalski and Braendle, 1956). The latter, however, presented evidence in good agreement with that of Bradley and Lederberg (1956), who attributed their results to the formation of heterokaryons and not synkaryosis. Much of the later work has been to determine whether genetic recombination or heterokaryosis was being observed (Bradley, 1957, 1958a, 1958b,

1959; Bradley, Anderson and Jones, 1959; Braendle and Szybalski, 1957, 1959; Sermonti and Spada-Sermonti, 1959). It was later recognized that recombination was largely responsible for earlier observations (Bradley, 1962).

Other work has indicated that mating type systems influence the genetic potential observed in species of Streptomyces. Bradley and Anderson (1958) found that certain combinations of growth-factor dependent mutants of Streptomyces coelicolor would not interact successfully to give prototrophic colonies. Such failures were found to be due to a compatibility system which controlled heterokaryon formation. This compatibility system was apparently determined by two factors, one of which probably arose by mutation. Supporting evidence for the existence of such compatibility systems has been presented by Braendle et al. (1959) and Bradley and Anderson (1958).

Hopwood (1959) takes up the problem of quantitative estimation of linkage and the mechanisms of recombination, and Bradley (1959) discusses the possible mechanisms which may play a role in the variability of Streptomyces species. Several factors, other than the environment, that influence variation in Streptomyces can be: 1) genetic composition of spores, 2) genetic composition of vegetative mycelia, 3) dissociation (Bradley, 1959). Hopwood (1959) suggests that discrepancies between re-

sults of different workers using different strains of Streptomyces coelicolor can be explained by assuming that strains referred to as S. coelicolor form a heterogeneous group of organisms. Within such a group, then, genuine strain-to-strain differences in behavior might not be unexpected.

Hyphae of the Streptomyces have generally been considered coenocytic (Klieneberger-Nobel, 1947; Webb et al., 1954; Hopwood and Glauert, 1960), and Bradley (1962) has pointed out that heterokaryosis as classically defined cannot occur in Streptomyces. This position, based on the excellent cytological work of Hopwood and Glauert (1958, 1960), results from the fact that the nuclear material is not confined in a discrete membrane. This lack of a discrete nuclear membrane, to this point unrecognized, has been the cause of much confusion concerning genetic interactions in Streptomyces. Even the coenocytic character of the hyphae also appears to have been challenged (Kogut et al., 1964) at least in Streptomyces griseus strain 3475 of Waksman.

Duggar et al., (1954) in a large scale study of Streptomyces species with respect to cultural and microscopic characteristics on a variety of media simply confirms and emphasizes the extent of the variability which is a characteristic of this group of organisms. The variability demonstrated is strong evidence that variations of this order exist and proceed essentially

along parallel lines, in nature and in culture. Hesseltine et al. (1954), Nyri (1961), and Sanchez-Marroquin (1962), in studies on methods useful for taxonomic purposes, indicate that the stability of biochemical or physiological characteristics within a species of Streptomyces is very good and a constant quantity. This may be true for a relatively small number of characteristics; but it is surprising in view of the fact that isolates of the genus have always presented difficulties from both morphological and cultural standpoints. Waksman (1957) concludes that, since the Streptomyces are known to be so temperamental, efforts should be made to maintain stock cultures by methods which preserve the original characteristics of the stock culture. He also emphasizes, as do others (Hesseltine et al., 1954; Lindenbein, 1952; Horvath, 1954), that such methods of preservation must be accompanied by a close adherence to bacteriological technique.

III. Materials and Methods

a.) Developing the Technique

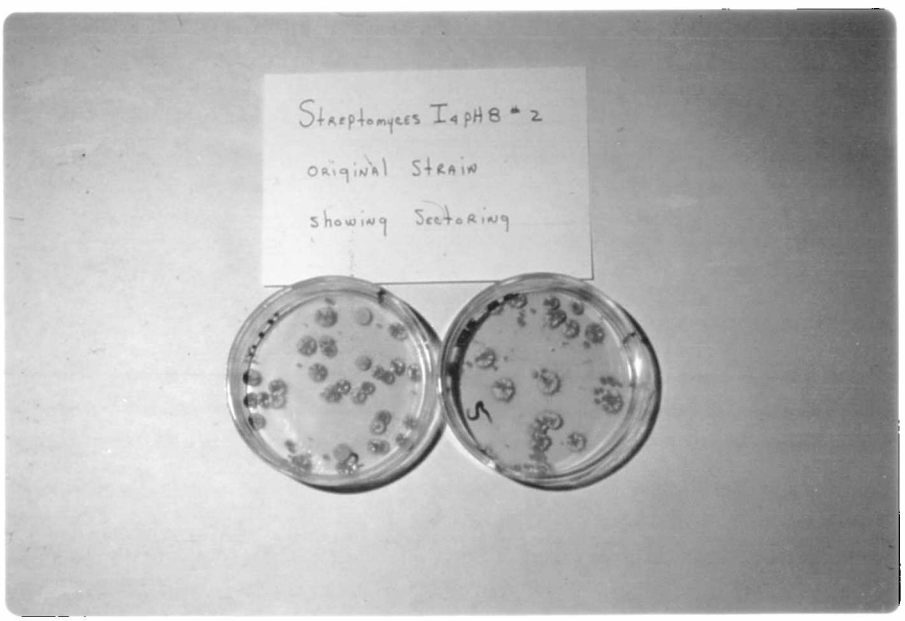
ORGANISM: The organism used in these studies is a variant of Streptomyces I_4 pH_8 #2. The parent strain is capable of utilizing cholesterol as the sole carbon source (Lewis, 1962; Davis, 1962), produces grey spores, and came from laboratory stocks.

Preliminary work demonstrated that colonies obtained by using spores as plating units were sectored. These sectored colonies produced both grey- and white-sporing areas (Plate I). By repeated subculture of the white-sporing areas, colonies producing only white spores were isolated. This variant utilized cholesterol well. The variant was designated Streptomyces I_4 pH_8 #2 (w), the "w" indicating production of white spores only.

Tests carried out on single colonies of the white-spore former, isolated from different plates, demonstrated the true-breeding character of the strain and its ability to utilize cholesterol.

MEDIA: A basal, phosphate-buffered medium, pH 7.0 (Table I), containing yeast extract or NH_4Cl as the nitrogen source and glucose as a carbon source, was used for all investigations involving technique (i.e., use of spores and/or mycelial fragments as plating units). The medium was prepared in three variations:

PLATE I



Streptomyces $I_4pH8\#2$: Original stock strain showing appearance of sectored colonies.

TABLE I

Composition per liter of media

Solution*	Component	M-9 Minimal Medium	Complete Medium
1.	KH_2PO_4	3.0gms.	3.0gms.
	Na_2HPO_4	9.85gms.	9.85gms.
	NaCl	0.5gms.	0.5gms.
	Distilled Water	600 mls	600 mls.
2.	$MgSO_4 \cdot 7H_2O$	0.2gms.	0.2gms.
	NH_4Cl	1.0gms.	2.0gms yeast extract
	Distilled Water	200mls.	200mls.
3.	Glucose	4.0gms.	4.0gms.
	Distilled Water	200mls.	200mls.
Final Volume		1000mls.	1000mls.

*Each solution is prepared and autoclaved separately for 15 minutes at 15 psi, 121 C. The solutions are then cooled and mixed as needed, final pH 7.0. Other Carbon sources are added to the minimal or complete system as desired.

1) Without agar for use in shaken cultures.

2) With 0.5% agar, for dilute-agar plates.

3) With 2.0% agar for sporulation and/or plating.

INOCULUM AND CULTURE CONDITIONS: Suspensions of spores or mycelial fragments in sterile distilled water were used as inoculum. Appropriate aliquots were pipetted into liquid media or spread confluently over agar plates with a sterile cotton swab.

Liquid cultures were incubated in a New Brunswick rotary shaker at 180 r.p.m. for six days at 27 C. Plate cultures were incubated in a 27 C dry-air incubator for six to fourteen days. Spore suspensions were prepared by scraping spores from cultures on the sporulation or dilute-agar medium. Sporulation on the dilute agar medium was very sparse. The spores were suspended in sterile distilled water and subjected to two minutes high speed treatment in a Waring blender microcup to effect uniform dispersion. The suspension was then filtered through sterile cotton wool or Whatman #1 filter paper to remove any mycelial fragments (as determined from slides stained with 1% Crystal Violet for one minute).

Mycelial suspensions from liquid, flask cultures were harvested after six days by filtration and washing with sterile distilled water. The mycelia were resuspended in sterile distilled water, blended two minutes as above to produce fragments of uniform size, and filtered through Whatman #1 filter paper.

Mycelia from dilute (0.5%) agar plates were prepared by first removing the spores (if any) with a damp sterile swab. A portion of all of the plate contents was then suspended in distilled water, followed by blending and filtration as described above.

Turbidity measurements were made on 5.0 ml. aliquots of the sample suspensions in a Klett-Summerson colorimeter with a #42 (blue) filter. Distilled water was used as a blank in all cases, since in the 0.5% agar preparations, even without filtration, background turbidity and viscosity could be ignored (Snider, 1963).

Twelve ml. samples of the appropriate suspensions (spore or mycelial fragments) were diluted in sterile distilled water to give a series of dilutions representing respectively 100% (undiluted), 75%, 50%, 25%, 12.5% and 6.25% portions of the original sample. After determination of turbidity on 5.0 ml. aliquots, serial, 1:10 dilutions were prepared from each tube to determine colony-forming-units (c.f.u.) one tenth ml. of the appropriate dilution was spread onto plates of complete and minimal medium. Duplicate or triplicate plates were prepared at three overlapping dilutions. The plates were then incubated at 27 C for three to five days and colonies counted on a New Brunswick Colony Counter.

Spore viability as a function of age was determined by inoculating 14 plates to obtain confluent growth. At various times two plates were removed; the spores were harvested and a suspension was prepared as described above. The turbidity was adjusted to a constant 100 Klett units and then the suspensions were serially diluted and the c.f.u./ml. determined (Figure 5).

b.) Investigations of Variability

MEDIA: The same basal medium used in the studies on techniques above was used for the studies of variability. The only nitrogen source used was NH_4Cl. Various carbon sources were added to this medium to determine their effect on the ability of the organism to utilize cholesterol.

CARBON SOURCES: CARBOHYDRATES: The carbohydrates used were prepared as 20% stock solutions, and filter sterilized in a Morton ultrafine sintered-glass bacteriological filter. On occasion the carbohydrates were pre-weighed and autoclaved dry at 110 C, 15 psi, for 20 minutes.

STEROIDS: The steroid carbon sources were pre-weighed in Waring blender cups, autoclaved dry at 121 C, 15 psi, for 10 minutes. Basal medium, with or without 2.0% agar, was then added and blended for two minutes at high speed (Peterson et al., 1962).

All carbon sources were added to a final concentration of 0.4%. Plates used to assay for cholesterol utilization con-

tained 0.1 to 0.2% cholesterol, in order to reduce the opacity which made colony enumeration difficult.

ASSAY FOR ABILITY TO UTILIZE CHOLESTEROL: Spores or mycelial fragments of the cholesterol-utilizing organism were harvested from the test media and plated onto complete, minimal and cholesterol medium. Complete medium contained yeast extract as nitrogen source and glucose as carbon source; minimal and cholesterol medium differed only in the source of carbon and NH_4Cl as nitrogen source (see Table I). The ratio of colonies formed on cholesterol medium to those formed on complete or minimal medium was used as a measure of variability. Five plates of each medium were inoculated at each dilution. It was observed that complete medium and minimal medium invariably gave the same average colony counts; this was used to indicate 100% capability of growth. The spores suspended in sterile distilled water were stored at 4 C and assayed as above from time to time to determine the effect of long term storage on the viable count and on the cholesterol/minimal (CHO/min) ratio.

PRODUCTION OF AUXOTROPHIC MUTANTS: Spores were harvested from four- to six-day old plates of complete medium; these were then washed and suspended in sterile distilled water. The suspension was blended for two minutes at high speed to separate spore chains and filtered as previously described. The turbidity was then ad-

justed to give a suspension of approximately 10^8 c.f.u./ml. Two 5 ml. aliquots were taken and treated as follows:

1) Serial dilutions were prepared and c.f.u./ml. determined.

2) The two aliquots were centrifuged at 6,000 r.p.m. for 15 minutes and the supernatant fluid discarded.

3) The contents of one tube were resuspended in 5 ml. of sterile distilled water as a control. The contents of the other tube were resuspended in 5 ml. of N-methyl-N-nitro-N-nitroso guanidine (Aldrich Chemical Company, Inc.) in 0.2 M citrate buffer at a pH of 5.0. This solution, containing 1 mg. NNG/ml., is self-sterilizing, but as an added precaution it was also filter sterilized.

4) 0.1 ml. aliquots were taken from the control suspension, and the treated suspension at zero time, and after eight hours (Table II and Figure 1).

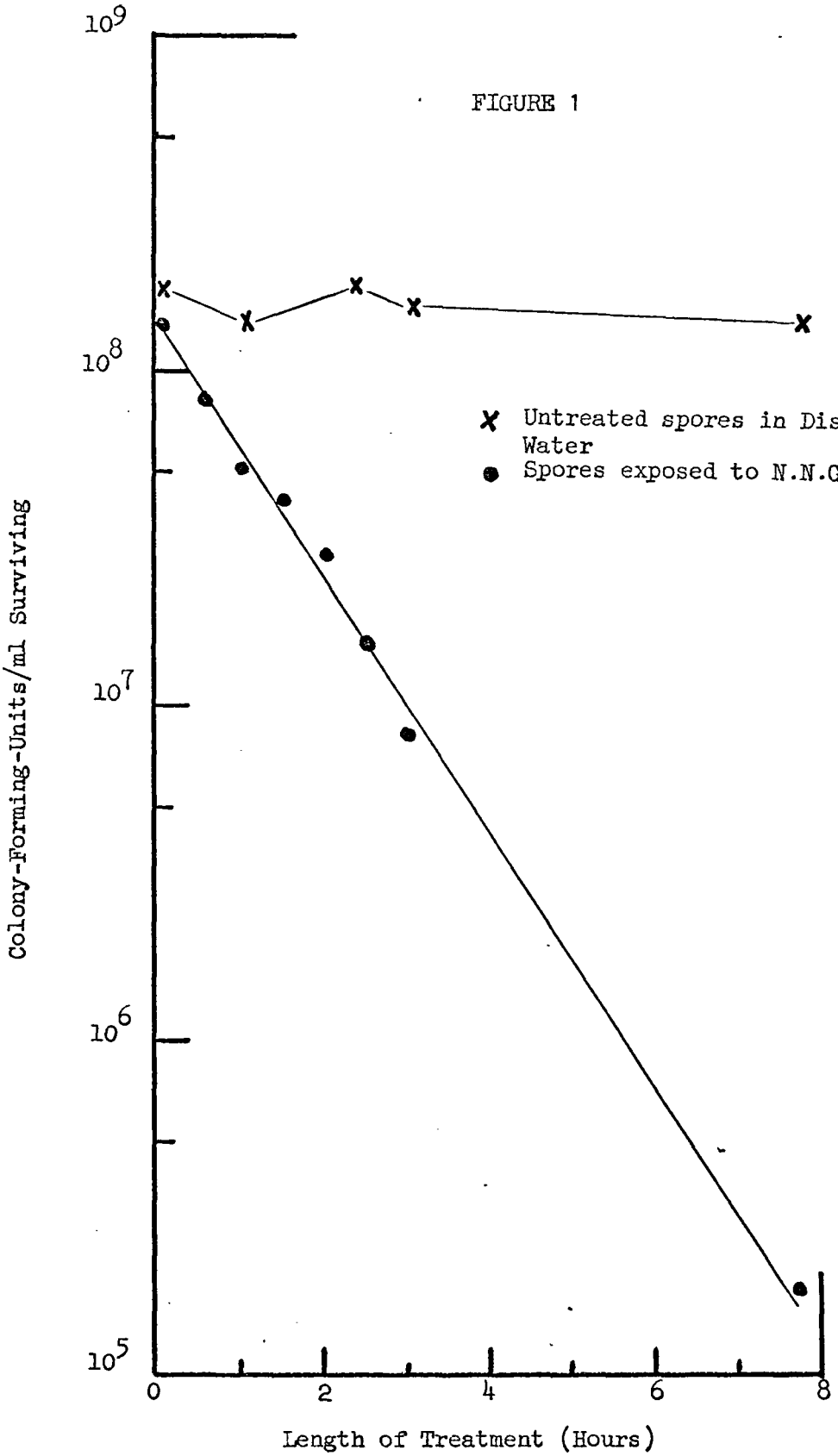
Detection of mutants was according to the method of Holliday (1956) with the replica plating technique of Lederberg and Lederberg (1952). Test substances were prepared as filter-sterilized stock solutions, concentrated such that 1 or 2 ml. gave the final desired concentration to 1 liter of medium (Table III). (Purines and pyrimidines 10^{-5}m, amino acids 10^{-4}m, vitamins 10^{-6}m).

TABLE II

Survival of Spores of Streptomyces I4pH8#2(w) as Colony Forming Units After Treatment with Mutagenic Agent N.N.G.

Time (Hrs.)	Sample Treated	Sample Citrate Buffer	Sample Distilled Water
0	1.2×10^8 C.F.U.	$1.05 \times 10^8 \frac{\text{C.F.U}}{\text{ml.}}$	$1.12 \times 10^8 \frac{\text{C.F.U.}}{\text{ml.}}$
7 Hrs. 45 min.	$1.8 \times 10^5 \frac{\text{C.F.U.}}{\text{ml.}}$	$1.4 \times 10^8 \frac{\text{C.F.U.}}{\text{ml.}}$	$1.2 \times 10^8 \frac{\text{C.F.U.}}{\text{ml.}}$

FIGURE 1



Survival curve of Streptomyces I_4pH8#2(w) spores exposed to N-Methyl-N-Nitroso Guanidine (1mg/ml).

TABLE III

Robin Holliday's 7 X 7 Scheme For 28 Substances

A	1 Guanine	2 Uracil	3 Adenine	4 Thymine	5 Cytidine	6 L-Arginine	7 DL-Valine
B	8 L-Cystine	2 Uracil	9 L-Leucine	10 L-Aspartic	11 L-Glutamic	12 DL-Alanine	13 DL-Threonine
C	14 DL-Serine	3 Adenine	9 L-Leucine	15 DL-Tyrosine	16 L-Proline	17 DL-Ornithine	18 DL-Tryptophan
D	19 L-Asparagine	4 Thymine	10 L-Aspartic	15 DL-Tyrosine	20 DL-Glutamine	21 Biotin	22 Folic Acid
E	23 Calcium Pantothenate	5 Cytidine	11 L-Glutamic	16 L-Proline	20 DL-Glutamine	24 Riboflavin	25 Nicotinic Acid
F	26 Inositol	6 L-Arginine	12 DL-Alanine	17 DL-Ornithine	21 Biotin	24 Riboflavin	27 Nicotinic Acid
G	28 Thiamine	7 DL-Valine	13 DL-Threonine	18 DL-Tryptophan	22 Folic Acid	25 Pyridoxine HCl	27 Nicotinic Acid

After Robin Holliday (1956) Nature, Lond. 178, 987.

n different media containing n different substances each. Thus requirements for $\frac{n(n+1)}{2}$ different substances can be distinguished.

Each media contains n substances according to a pattern which provides for each substance occurring in one medium only, or in two media, the two being different in all other aspects.

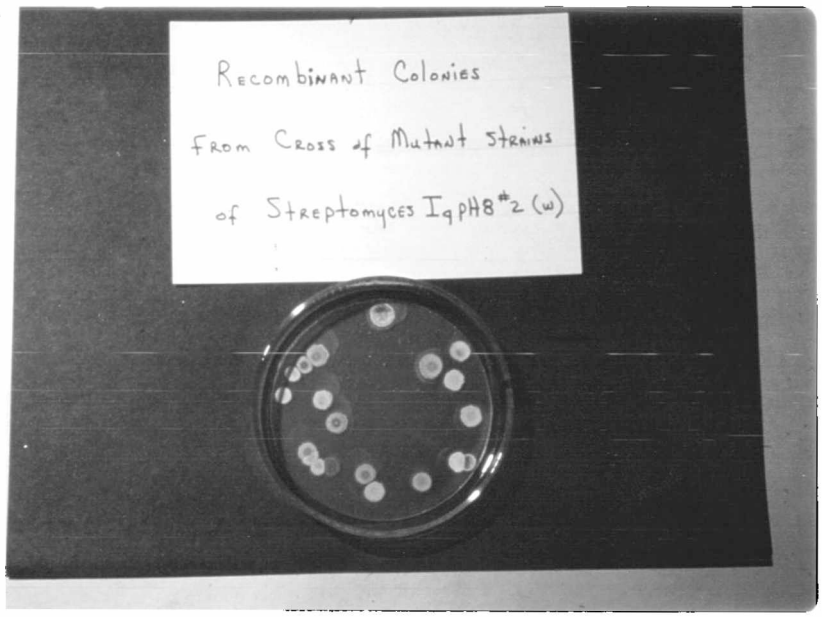
RECOMBINATION EXPERIMENTS:

Mutant organisms were plated in mixed culture on complete medium (i.e., complete agar plates were streaked to give confluent growth with cotton swabs containing first one mutant and then the other). The plates were incubated for six days at 27 to 30 C. Spores were then harvested and washed and plated to minimal to complete and to the appropriate supplemented diagnostic media. Suspected recombinant colonies appearing on unsupplemented minimal medium after six days at 27 to 30 C were verified as recombinants according to the criteria of Hopwood, Sermonti and Spada-Sermonti (1963) which show that haploid recombinant colonies breed true on subculture, whereas segregating the heterozygotes will produce spores having the characteristics of one or the other parent strain and will not grow on minimal medium (Tables IV and V).

CHOLESTEROL PURIFICATION: Commercially available cholesterol was purified by precipitation as the acetate from acetic acid. This precipitate was washed with methanol, and the crystals were dried overnight in a hood. After the preliminary drying the cholesterol was dried to a constant weight in a 90 C oven and the melting point (149.5 to 150 C) determined.

Flasks of liquid, cholesterol medium containing 100 mg. purified cholesterol/100 ml. of medium were inoculated with

PLATE II



Recombinant Colinies Resulting From Mixed Growth of Arginine and Adenine Deficient Auxotroph of Streptomyces I_4 pH_8 #2 (w)

TABLE IV

RECOMBINATION EXPERIMENT

Demonstration of Recombination Between Adenine Deficient and Arginine Deficient Auxotrophs of Streptomyces $I_4pH8\#2(w)$

Diagnostic Plating Medium	Parental Phenotype $ADE^- \times ARG^-$
Complete	1.3×10^8 C.F.U./ml
Minimal	*5.0×10^5 "
Minimal + Adenine	1.2×10^8 "
Minimal + Arginine	2.7×10^7 "

* Apparent Recombinants

TABLE V

Confirmation of 10 Recombinants Isolated from Experiment in Table IV

Colony Number	Diagnostic Medium		
	Minimal	Arginine	Adenine
1	*+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+

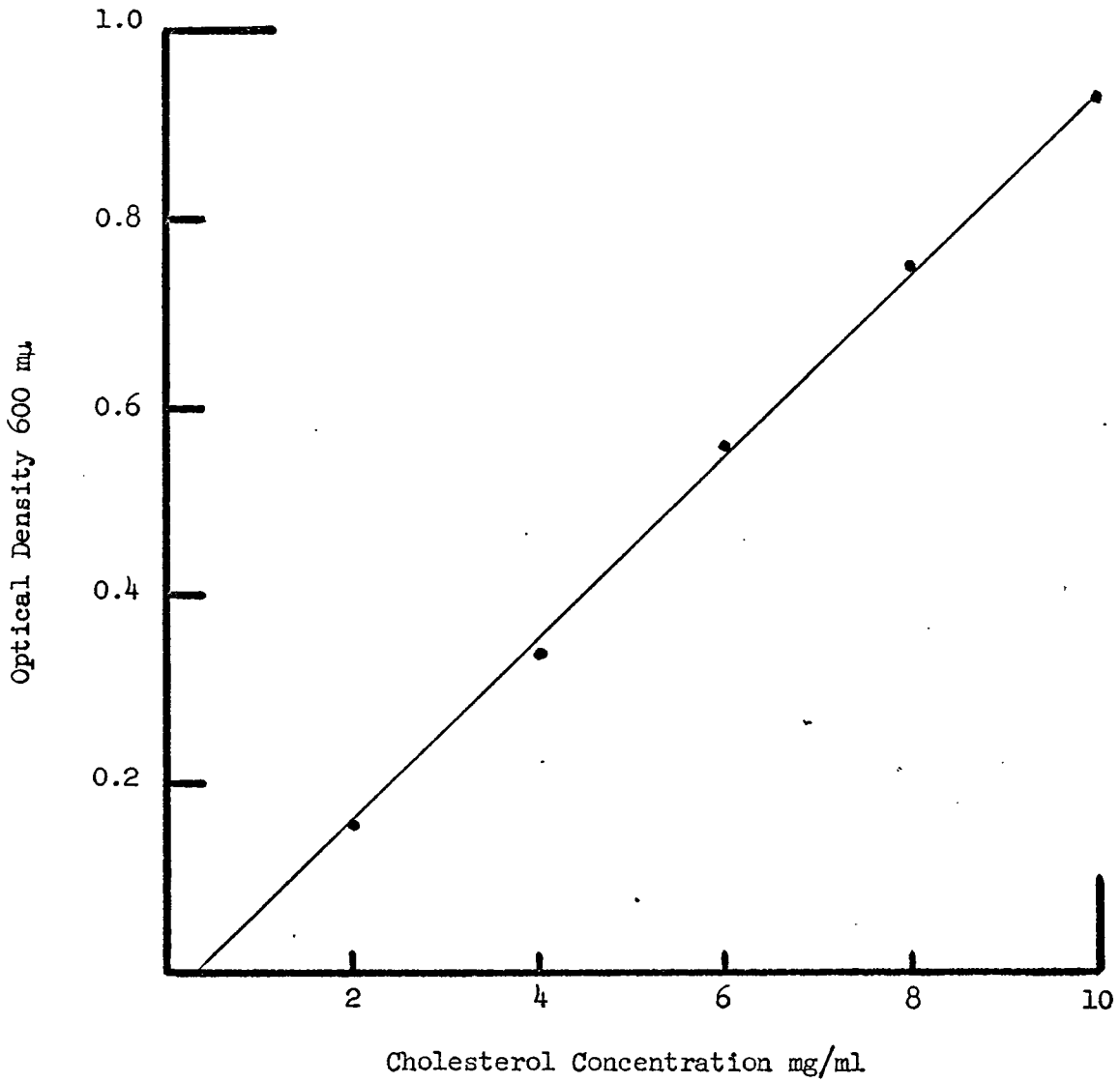
*Strong growth obtained on all three diagnostic media.

spore suspensions, 1 ml./flask, to determine the efficiency of cholesterol utilization of suspected recombinants, wild type organism and the auxotrophic mutants. The inoculated flasks were harvested at day zero and at four-day intervals thereafter. Each flask was extracted with 10 ml. of chloroform and 0.1 ml. aliquots of such chloroform extracts used for determination of residual cholesterol.

Residual cholesterol was determined by the method of Zurkowski (1964), which utilizes only a single reagent and eliminates the need for lengthy evaporation procedures (see standard curve, Figure 2).

FIGURE 2

Determination of Residual Cholesterol
Calibration Curve.



IV. Results and Discussion

a.) Developing the Technique

Snider (1963) proposes five major variables which affect the use of macerated mycelia in the fungi:

1) The total concentration of hyphal fragments

2) The proportion of viable fragments

3) The frequencies of the genetic types of fragments

4) The number of nuclei per fragment

5) The spatial distribution of nuclei among fragments

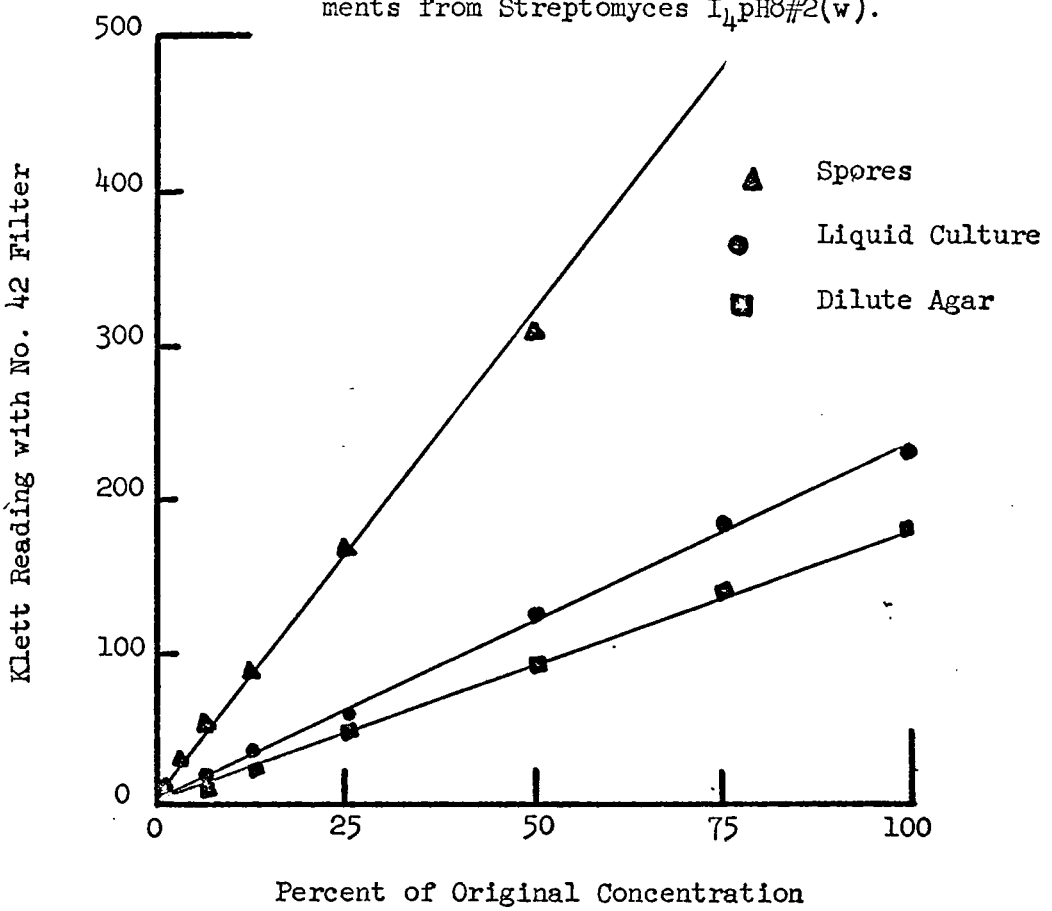
Maceration is thought to have no effect on the nuclear ratio, since it is assumed that such treatment destroys cells randomly, although this may not be true in every case. Since we are not considering nuclear ratios here, and since we are using only one genetic type of organism, the variables 3 and 5 may be conveniently ignored.

Turbidity is linearly related to concentration for a homogeneous suspension of minute, ideal particles, as such turbidity is a measure of the total concentration of fragments or spores, both viable and non-viable.

Figure 3 shows calibration curves displaying the relationship between turbidity and serial 1:2 dilutions of spore and mycelial suspensions, expressed as a percentage of the original concentration. A strict linear relation exists between T values

FIGURE 3

Turbidity vs Percent of Original Concentration: Spores and Mycelial Fragments from Streptomyces I_4pH8#2(w).



10-200 in the suspensions of mycelial fragments and up to 310 T units in the spore suspension. Above these limits a sharp deviation occurs.

The relation between turbidity and concentration, the latter expressed as colony-forming-units per ml., is shown in Figure 4. Approximately the same degree of linearity is again apparent. The disparity in concentration between spore suspension and mycelial suspensions at the same turbidity values, points up the fact that the spores are much smaller than mycelial fragments. This is one advantage of spore suspensions over mycelial fragment suspensions. The spores being smaller more nearly approach the concept of the ideal particle.

One can now calculate a viability factor (f), defined by Snider (1963) as, "The number of viable fragments per unit turbidity per unit volume in milliliters of macerate." The factor is determined from counts of mycelia that grow on plates of complete medium

(f), can be related to the total concentration of fragments or spores (viable and non-viable) expressed as turbidity by use of a simple equation, as follows:

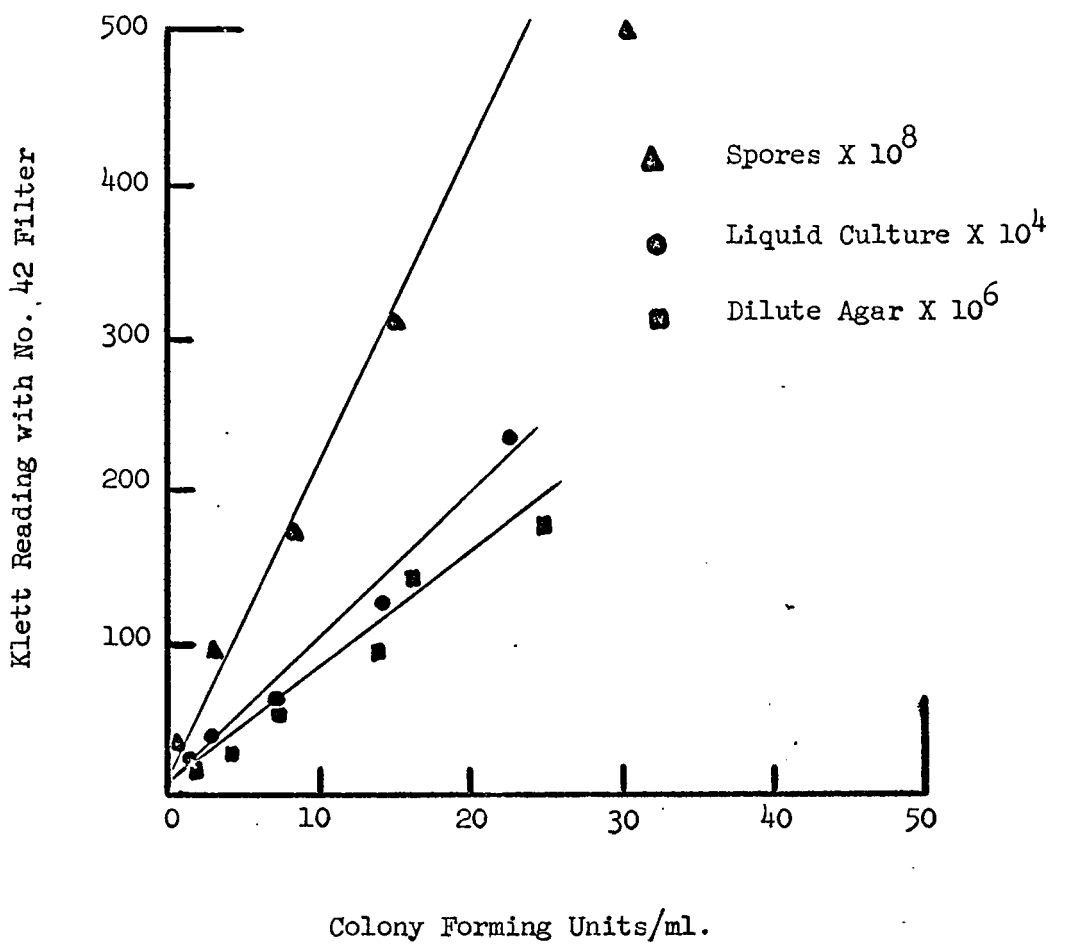
$$F = f \cdot T \cdot V \quad \text{(from Snider, 1963)}$$

where:

F = total number of viable fragments contained in

FIGURE 4

Turbidity vs Colony Forming Units per ml.
Spores and mycelial fragments from Streptomyces I4pH8#2(w)



any volume (V) for observed values of (f) and (T).

V = volume of macerate in ml.

T = turbidity (total concentration of fragments, viable and non-viable)

f = viability factor

Calculated values for the viability factor (f) are reproducible under the same conditions, for the strain of organism under consideration (Table VI). Other variables such as age of culture, speed and duration of maceration, however, affect the viability of mycelial suspensions. With spores, (f) is relatively constant with respect to the age of the culture. Storage of suspensions at 5 C appeared to have little effect, but suspensions were never refrigerated more than 24 hours before use. In the case of long-term storage of spore suspensions, a definite decline can be noted.

With the use of the viability factor, one can readily calculate the dilution of known numbers of viable fragments for plating on various media. The turbidity of unfiltered suspensions of mycelial fragments, plotted against dry cell weight, can be used as a standard curve in growth experiments (Guidry and Trelles, 1962).

FIGURE 5

Spore Viability vs Culture Age at Constant Turbidity

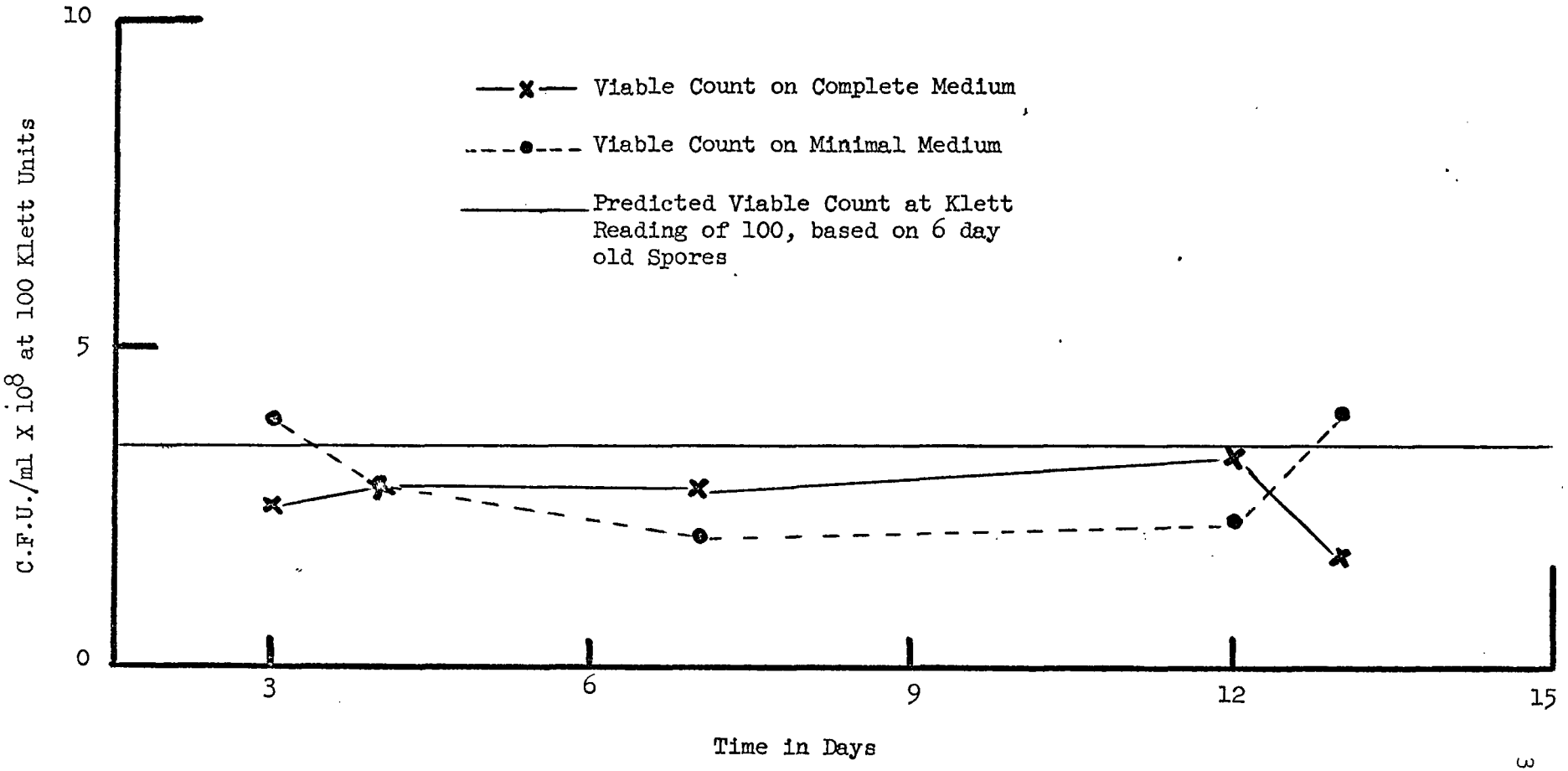


TABLE VI

Comparison of Viability factor "f" as defined by F=f.T.V

Percent concentration of sample	Source of Sample		
	Fragments Liquid Culture	Spore Suspension	Fragments Dilute Agar
100	0.94×10^3	5.8×10^6*	1.2×10^5
75	1.50×10^3 *	----	1.1×10^5
50	1.10×10^3	4.8×10^6	1.5×10^5
25	0.85×10^3	4.6×10^6	1.5×10^5
12.5	0.80×10^3	2.8×10^6	1.7×10^5
6.25	1.20×10^3	1.6×10^6	1.2×10^5
3.125	----	1.0×10^6	----
1.06	----	1.1×10^6	----
Average	0.98×10^3	3.0×10^6	1.4×10^5

* Not included in average because not on linear portion of curve (Turbidity vs C.F.U.

b.) Recombination Experiments

Two auxotrophic mutants of Streptomyces I_4 pH_8 #2 (w) were isolated and characterized as described. One mutant was shown to require adenine and the other arginine. The arginine requiring mutant had characteristics similar to a class of ornithine cycle mutants described by Bonner (1946) in Penicillium notatum.

Recombination between the two auxotrophic strains could be demonstrated by mixed growth experiments. Table IV shows the results of one such experiment. Haploid recombinant colonies are homogeneous and breed true on subculture (Hopwood et al., 1963). Ten apparently recombinant colonies were chosen at random for testing. All ten colonies were shown to be true recombinants rather than "heteroclones" as described by Sermonti et al. (1960), (see Table V). In genetic studies with Streptomyces species, the two types of colonies, recombinants and heteroclones, may arise together representing 1 in 10^2 to 10^5 of the spores (or other colony-forming-units) plated.

Nearly all of the spores arising from "heteroclones" as opposed to recombinant colonies are haploid, following segregation of the heterozygous nuclei during growth (Hopwood et al., 1963). Such spores have one or the other parental growth requirement and are unable to grow on deficient media (see Table

V). These characteristics provide an easy means of recognizing such colonies and differentiating them from truly recombinant colonies.

Interest in heteroclones lies principally in the fact that in them the heterozygous condition of the nucleus is prolonged enough for its segregation to be studied (Hopwood, et al., 1963). Our studies were principally concerned with demonstrating recombination between auxotrophic strains of a rather unique Streptomyces species. Once this was accomplished it was necessary to show that such mutants and the resultant prototrophic recombinants were incapable of utilizing cholesterol as efficiently as the "wild type" original strain. Spores from the auxotrophic strains, prototrophic recombinants and the original strain were harvested from complete medium, dispersed in distilled water, adjusted to approximately the same optical density, and inoculated into cholesterol-minimal salts medium (without agar) and the amount of cholesterol utilized in four days measured. Table VII shows the results of one such experiment and provides evidence that the existence of such auxotrophic recombinants can effect the ability of the organism in question to utilize cholesterol. Such recombinants appearing as a result of storage or growth on various other carbon sources could result in the disappearance of the cholesterol utilizing characteristic altogether unless specific traits were checked frequently.

TABLE VII

Cholesterol Utilization Experiment: Wild Type Organism, Mutants, and Recombinants of Streptomyces I4pH8#2(w) grown in Liquid Minimal Medium Plus 100mg Cholesterol for four days at 30°C

	0 Day	4 Days
Organism	Residiual Cholesterol (Avg.)	Residiual Cholesterol (Avg.)
Controls	86mg/100ml	84mg/100ml
Adenine Mutant	86 "	64 "
Arginine Mutant	86 "	62 "
Wild Type Organism	86 "	42 "
Recombinant	86 "	62 "

c.) Carbon Source and Cholesterol Utilization

The experience of Lewis, Davis, Brown, (Personal Communication) and others from this laboratory demonstrated that loss of the cholesterol-utilizing factor could be remedied by a period of enrichment growth on various organic media, e.g., Bennett's agar, tomato paste agar, oatmeal agar, etc. From these considerations it would seem that this physiological character might be dependent upon or would be affected by changes in the organic carbon source.

Studies were undertaken with liquid cultures, spores and fragments from dilute agar plates. It was shown that the most dramatic decrease in cholesterol-utilizing efficiency could be demonstrated with mycelial fragments from liquid culture (Table VIII). It was necessary, however, to show that the method chosen for assay as described in materials and methods actually was representative of the cholesterol-utilizing efficiency. Table IX shows the results of such an experiment and indicates that expression of the assay as % cholesterol utilization represents a true picture of the experimental condition.

Studies carried out with various sugars in liquid culture show that the efficiency of cholesterol utilization rises to a maximum at about 48 hours and thereafter declines. Any mycelium harvested after 96 hours and used as inoculum may lose

Table VIII

Comparison of Cholesterol Utilizing Ability of Spores and Mycelial Fragments

Direct Plating onto Complete and CHO Media, Average Concentration

Sample	Complete	CHO	* % CHO Utilization
Liquid Culture Fragments	3.6×10^5	9.7×10^4	26
Spores from dilute agar plates	4.1×10^6	3.9×10^6	95
Fragments from dilute agar plates	9.2×10^4	8.5×10^4	92

* Ratio of colonies formed on cholesterol medium to those formed on complete or minimal medium..

TABLE IX

Direct and Replica Plating: Complete and CHO Media

Direct Plating

Complete	CHO	% CHO
3.9×10^7	3.6×10^7 (Average of 5 plates)	90

Replica Plating

Original on Complete Medium	CHO	Minimal	% CHO
48 Colonies	37	41	90
27 "	14	14	100
31 "	24	27	88
60 "	46	48	95
33 "	27	30	90
Average: 39	29	32	92

TABLE X

Tabulation of CHO/Min or Cholesterol Efficiency

	0 Time	48 Hrs.	96 Hrs.
Minimal Salts	94%	100%	71%
Minimal + Glucose	94%	83	55
Minimal + Lactose	94%	107	58
Minimal + Maltose	94%	106	55
Minimal + Sucrose	94%	110	42
Minimal + CHO	94%	100	71

the ability to utilize cholesterol. If carried on over several transfers without determination of the cholesterol-utilizing efficiency (Table X) the consequent loss of this characteristic would be difficult to interpret and would certainly invalidate any experiment based on cholesterol utilization.

Other carbon sources affected the ability of growth, from the spores or mycelial fragments harvested from them, to utilize cholesterol (Table XI). These results are consistent with the data of Davis, et al. (1962), who found two enzymes for the degradation of cholesterol; one was constitutive and the other inducible. Thus, a certain percentage of spores and fragments would be constitutive for one enzyme and their number would be a background count as it were. Growth upon other substances would perhaps induce the production of the other enzymes, resulting in an increase in the number of cholesterol-utilizing spores or fragments over the number designated as background above. In a number of cases this was shown to be so for on replica plating from complete, to minimal, and to cholesterol media there appeared to be colonies capable of growing on all three, and others capable of growth both on minimal and cholesterol, and still others capable of growth on minimal only.

The results with various carbon sources are consistent with the idea that genetic mechanisms greatly affect the final

TABLE XI

Effect of various Carbon Sources on Cholesterol Utilization*

Carbon Source	Spores	Mycelial Fragments	% Cholesterol Utilization
None		+	84
Lactose	+		99
Maltose	+		66
Sucrose	+		88
Glucose	+		52
Cholic Acid		+	100
Sitosterols		+	94
Δ-5-Cholestene		+	73

* Ratio of the number of colonies on cholesterol medium to those developing on minimal medium.

efficiency of a culture for a subsequent substrate, especially if the subsequent carbon source is not the most readily usable or the most abundant.

Mortlock, et al. (1964) studied a combination of circumstances in Aerobacter aerogenes which amount to a new mechanism for growth on rare or uncommon substrates. Basically they account for the mechanism by involving selection of mutants constitutive for enzymes capable of rendering the uncommon substrate readily usable. With such a concept one could explain the capability to metabolize uncommon substances without resorting to the concept that genetic information exists for the synthesis of unique enzymes which attack substrates rarely encountered in nature.

With respect to spore suspensions our work has shown that such suspensions when stored at 4 C present an excellent method for preservation. Gadó, et al. (1961) demonstrated that spore suspensions of Streptomyces rimoses BS-21 kept at -10 C are suitable for inoculation and also for preservation of the species. Loss of physiologic activity has been found to parallel to some extent the serial number of subcultures of the organism and also duration of storage.

We have kept a spore suspension at 4 C for over a year and the number of colony forming units on complete medium has

not decreased to any great extent but the number of colony forming units which will utilize cholesterol has fallen from 90% to 79%. Three colonies incapable of utilizing cholesterol have been isolated and used to produce spores for inoculum. The resulting spore suspensions of the three cholesterol non-utilizers were incapable of utilizing cholesterol in minimal salts medium at the end of six days, however, if first grown in complete liquid medium, the resulting mycelial fragments have 94% cholesterol-utilizing efficiency. Such results indicate that, on storage, spores tend to mutate spontaneously or to lose some precursor or priming material necessary for their immediate activity on cholesterol as a carbon source. A deficiency in hydrogen donors such as fumarate or succinate might account for such a phenomenon. Further studies based on cholesterol utilization, in the presence of trace amounts of succinate or fumarate, would need to be undertaken to clarify the situation.

V. SUMMARY

1. The use of mycelial fragments derived from cultures of Streptomyces I_4 pH_8 #2 (w) as plating units as well as inoculum has been investigated and confirmed.

2. Two mutants of Streptomyces I_4 pH_8 #2 (w) were isolated using nitrosoquanidine as mutagenic agent.

3. Recombination has been demonstrated in a rather unique strain of Streptomyces.

4. Recombination has been demonstrated to be a possible factor in the variability of Streptomyces I_4 pH_8 #2 (w).

5. Storage of spores and their subsequent use as inoculum or as plating units has been shown to play a role in the variability of Streptomyces I_4 pH_8 #2 (w). Loss of the ability to utilize cholesterol on storage makes necessary careful check procedures to assure presence of any particular physiological trait.

6. It has been demonstrated that growth on various carbon sources other than cholesterol affects the subsequent ability of the organism to utilize cholesterol.

7. The causes of variability in this organism and in Streptomyces in general has been discussed. Spontaneous mutation and deficiency of precursors have been discussed as possible causes of variation in this organism and in the Streptomycetes in general.

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