

THE EFFECTS OF DIMETHYL SULFOXIDE  
ON LIPIDS OF  
ASPERGILLUS NIGER

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A Thesis  
Submitted to  
The Department of Biology  
Kansas State Teachers College, Emporia, Kansas

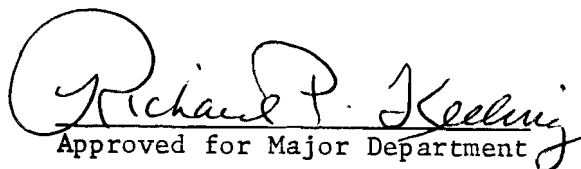
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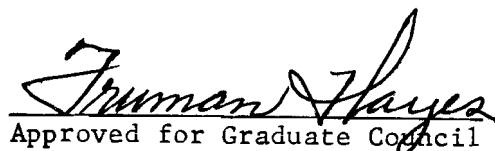
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## INTRODUCTION

Dimethyl sulfoxide (DMSO) was first prepared by Alexander Saylzeff in 1886, and it is now prepared from a common waste product from paper manufacture. It is useful in preserving animal and human tissue in that it lowers the freezing points of biological fluids (Schiller, unpublished, 1967).

The penetrating properties of DMSO led to its usefulness in aiding nutritive element transport in plants, and in the control of plant diseases (Garren, 1967; Keil, 1965).

The discovery of its penetration through the skin led to a potential clinical usefulness. It seems to have broad but mild biological activity. It may induce the release of histamine, has antiinflammatory power, is analgesic, bacteriostatic, diuretic, and sedative. It also has low toxicity on single or repeated application. It can induce a "garlic breath" and occasional allergic reactions as well as cause drying of the skin to which it is applied due to its hygroscopic nature in concentrations above 50 percent, vol/vol (Leake, 1967).

Clinically, it is reported to be helpful in treating rheumatoid arthritis (Matsumoto, 1965), and mild inflammatory conditions (Steinberg, 1967). Also, it may aid steroids to penetrate the skin (Steinberg, 1967). Because of reports that pathological changes in the eyes of experimental animals occur after treatment with DMSO (Wood et al, 1967; Kleberger, 1967),

the United States Food and Drug Administration blocked further clinical research with DMSO.

It has been demonstrated that the configuration of certain enzyme proteins is altered in the presence of DMSO, and this alteration is reversed when DMSO is removed. The effectiveness of DMSO in this regard may be related to its size and capacity to substitute for water, suggesting a possible mode of action in living systems (Rammler and Zaffaroni, 1967).

That DMSO can serve as a metabolite has been demonstrated with Eschericia coli which can reduce DMSO to dimethyl sulfide (DMS) through the action of a specific enzyme (Ando, 1957).

The effect of DMSO on fungi was first observed by Teel (unpublished, 1965). The visible effect consisted of the bleaching of the characteristic color from the conidia of the dark pigmented members of the Aspergillus-Penicillium complex. It was also found that the "spines" normally seen on the conidia of Aspergillus niger failed to appear in the presence of DMSO. Such effects were apparently temporary because when these same conidia were used to inoculate DMSO-free media, the natural dark pigment and spines appeared in the conidia of the resultant colonies.

These effects on the morphology of fungi encouraged the studies included in this paper. A biochemical approach was used in order to determine whether or not metabolic pathways of Aspergillus niger were altered by DMSO.



The specific study of lipids of Aspergillus niger was undertaken initially because of a suggestion by Dr. J. N. Shively of the University of Nebraska, Lincoln, Nebraska. From his own investigation of the lipid and phospholipid metabolism of bacteria, he reasoned that lipid metabolism of these fungi might well be affected by DMSO.

## METHODS AND MATERIALS

### Experimental Stock

All experiments were conducted with Aspergillus niger (NRRL 272). The initial culture was obtained from the mycology collection maintained at Kansas State Teachers College. This initial culture was subcultured as the organism was needed.

### Media and Concentrations of DMSO

The organism was grown on either Czapek Solution Agar (Difco Laboratories--0339-01) or Czapek Dox Broth (Difco Laboratories--0338-01) depending on whether an agar or broth medium was required.

DMSO-amended media contained 1% concentrations of DMSO (vol/vol). Any other materials introduced into the media were in the same concentrations as DMSO.

### The Preparation of Inocula

Fifty ml agar was placed in a 250 ml Erlenmeyer flask and inoculated from a slant culture with a sterile loop. When the organism completely covered the surface of the agar, 50 ml broth was aseptically poured into the flask to suspend the conidia. This procedure was followed in order that all media in a particular experiment were inoculated in a standard fashion.

### Introduction of Materials into Media

All materials were introduced into the media by sterile

syringes, and the media were inoculated by the same method on the basis of 1 part inoculum/100 parts medium (inocula was placed on agar in amounts that barely covered the surface of the agar).

Throughout the experiments glucose, glycerine, DMSO, Sudan Black B, or any combination of these was added to the media. Glucose and glycerine were added before autoclaving. However, DMSO and Sudan Black B were added after autoclaving because of their volatility and heat lability.

Immediately after autoclaving, containers of agar were placed in a 70° C water bath for about 30 minutes which allows the agar to remain in a liquid state without altering heat labile materials significantly when they are introduced into the media. Broth was allowed to cool before labile materials were introduced.

#### Sudan Black Staining

Sudan Black B (Fisher Scientific Co--26150) is a reagent used to stain lipids in cellular structures, and it was reasoned that Sudan Black could be used as an indicator of lipid concentration within the organism by allowing the organism to absorb the stain from the growth medium.

The following experiment was designed to determine the effect of DMSO on lipid concentration, and, the consistency of this effect in the presence of two lipid precursors, glucose and glycerine.

Fifty ml agar was placed in each of ten 125 ml Erlenmeyer flasks. The flasks were numbered and the media were amended with additional ingredients according to the following protocol:

- Flask No. 1 - Agar (Control or normal medium, no additional ingredients)
- Flask No. 2 - Agar, DMSO (DMSO-amended control medium)
- Flask No. 3 - Agar, Sudan Black B
- Flask No. 4 - Agar, Sudan Black B, DMSO
- Flask No. 5 - Agar, Sudan Black B, glucose
- Flask No. 6 - Agar, Sudan Black B, glucose, DMSO
- Flask No. 7 - Agar, Sudan Black B, glycerine
- Flask No. 8 - Agar, Sudan Black B, glycerine, DMSO
- Flask No. 9 - Agar, Sudan Black B, glucose, glycerine
- Flask No. 10 - Agar, Sudan Black B, glucose, glycerine, DMSO

The flasks were inoculated and incubated for two weeks at 27° C, after which samples of conidia and mycelia were observed under a microscope. Observations were confined to the comparison of color intensity in the conidia and mycelia. To insure that differences in color intensity were due to changes in lipid concentration rather than changes in light intensity, all samples were observed under the same light conditions which were checked with a light meter as each sample was observed.

#### Mycelial Analysis

This experiment was designed to determine whether or not DMSO alters lipid synthesis in the mycelial portion of the organism, and to determine whether or not significant changes occur in the presence of two lipid precursors, glucose and glycerine.

One-hundred ml broth was placed in each of eight 500 ml Erlenmeyer flasks. The following protocol was used:

- Flask No. 1 - Broth (Control or normal medium,  
no additional ingredients)
- Flask No. 2 - Broth, DMSO
- Flask No. 3 - Broth, glucose
- Flask No. 4 - Broth, glucose, DMSO
- Flask No. 5 - Broth, glycerine
- Flask No. 6 - Broth, glycerine, DMSO
- Flask No. 7 - Broth, glucose, glycerine
- Flask No. 8 - Broth, glucose, glycerine, DMSO

The flasks were inoculated and placed on a rotary action shaker (set at a shake frequency of 200 per minute) for a period of six days. The mycelial pads were harvested on Whatman No. 1 filter paper and placed in a 100° C oven until the filter paper was dry to the touch. The mycelial pads were taken from the drying oven and transferred to a vacuum dessicator for a period of 48 hours. They were then transferred to pre-weighed aluminum tares and weighed on an analytical balance.

The dried pads were collected in culture tubes containing Folch extraction medium (chloroform: methanol 2:1 vol/vol, 0.1 gr tissue/1.0 ml (Packer, 1967), layered with helium, and placed in a freezer for 2½ days at -68° C.

Twenty microliters of each extract was spotted on thin-layer chromatography plates spread with Silica Gel G (Research Specialities Co--8067) which were developed for 70 minutes in petroleum ether/diethyl ether/acetic acid 90:10:1 vol/vol (Malins and Mangold, 1960).

The spots were detected by first spraying the plates with 50% H<sub>2</sub>SO<sub>4</sub> and then charring them in a 115° C oven for 30 minutes (Bingham and Kurtz, 1966).

The chromatograms were recorded on photographic paper (Kodak--F-4) by placing the plates (absorbant side up) on top of the paper and exposing to light from a photographic enlarger. When the paper was developed in the traditional fashion, the spots appeared white against a dark background (Plates 3 and 4) with resolution comparable to the original plates (Seher, 1959).

#### Analysis of Conidia

The procedure for the analysis of spores was the same as that described for the analysis of mycelial pads except an agar medium was used in place of a broth medium. The agar was dispensed in Rhoux bottles (Plate 1) and the drying procedure was eliminated.

The conidia were harvested from the bottles by a special device (Plate 1) constructed from a cellulose membrane filter holder (Metricel, Ga-6 grid, 0.45 micron pore size, 1 inch diameter, Gelman Instrument Co., Scientific Products--F2932 1). A one-eighth inch diameter glass tube was cut the length of the bottle, and bent at one end. The other end was attached to the inlet opening of the holder, and the outlet end of the holder was connected to a vacuum line. With this device the spores were harvested efficiently within a short period of time.

Lipid extracts were prepared from the conidia and analyzed according to the same procedures described in the analysis of mycelial pads.

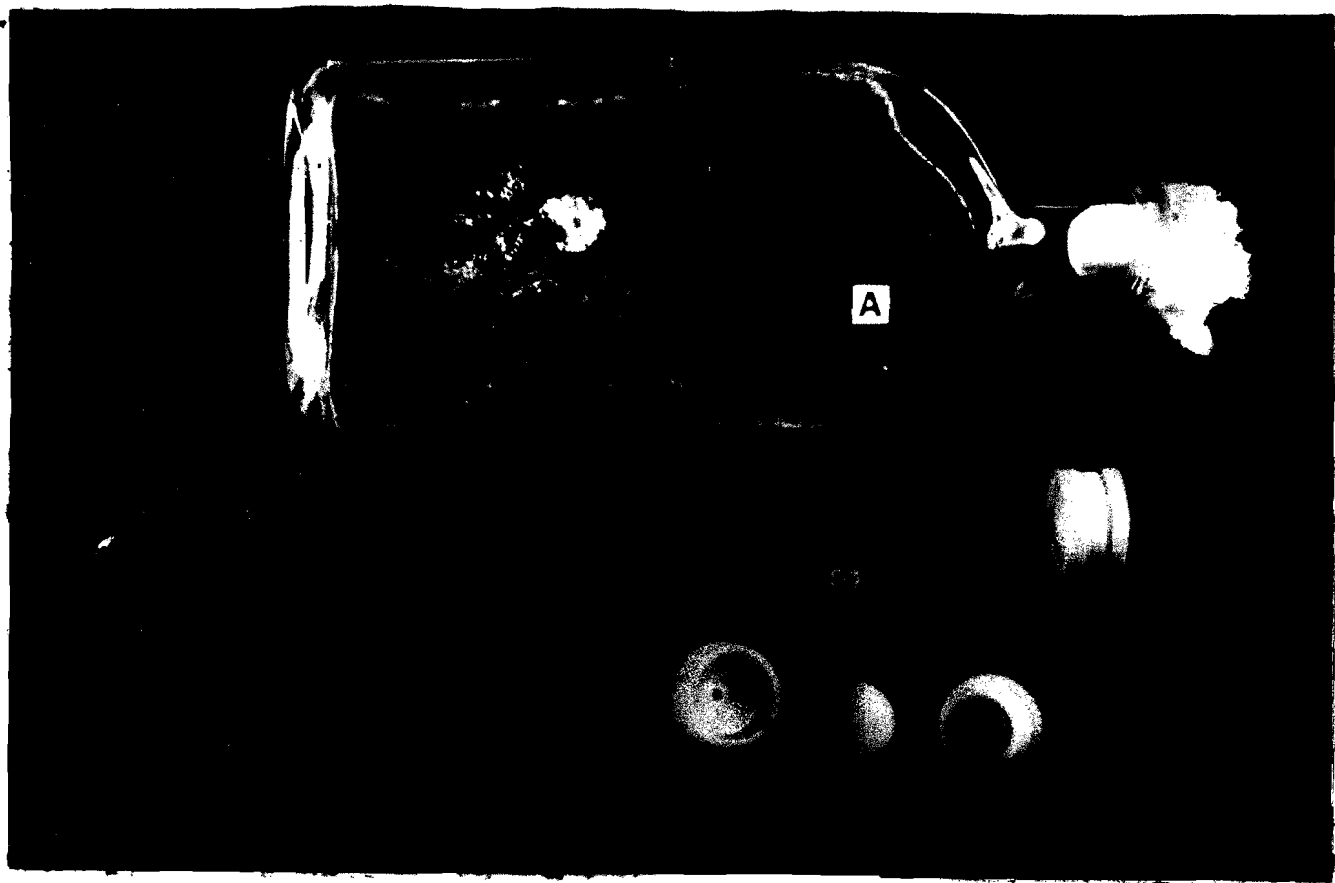


Plate 1. (A) Rhoux bottle used to cultivate fungus for conidium analysis; (B) Spore harvester constructed from a cellulose membrane filter holder; (C) Exploded view of cellulose membrane filter holder.

## RESULTS AND DISCUSSION

Conidia from DMSO-free media, stained with Sudan Black, were darker in color than those conidia from the control medium (Plate 2, Table I). This indicates that Sudan Black stained the lipids that were present.

The color intensity was equivalent in conidia from DMSO-free media amended with Sudan Black, glucose, glycerine, or a combination of the three. This indicates that these lipid precursors, glucose and glycerine, did not significantly affect lipid concentration in the conidia.

Conidia from DMSO-amended media were bleached (Plate 2, Table I). Sudan Black failed to appear in conidia from stained media because the lipids with which it reacted were not present. Since lipids were not present in conidia from DMSO-amended media, DMSO apparently inhibits the synthesis of these lipids regardless of the presence or absence of the two lipid precursors, glucose and glycerine.

In examining the chromatogram of lipid extracts from conidia (Plate 3), it was found that one spot appeared in extracts from conidia harvested from DMSO-free media. This occurred in spite of the presence or absence of glucose and glycerine or a combination of these.

These results correlate with results from Sudan Black staining. That is, Sudan Black did not stain conidia from



DMSO-amended media, nor did spots appear on the chromatogram where conidia were harvested from DMSO-amended media. This further indicates DMSO inhibits lipid synthesis in conidia.

The mycelia (Table I), regardless of the media on which they were grown, were not stained by Sudan Black. On the thin-layer chromatogram (Plate 4, Table II) spots appeared in the same pattern in extracts from mycelia harvested from all types of media.

This indicates DMSO has no effect in the vegetative part of the organism. Also Sudan Black did not stain the mycelia, and yet some forms of lipids appeared on the chromatogram. It was apparent that DMSO affected only those lipids stained by Sudan Black in the conidia.

It is possible then, that the morphological changes (bleaching of the pigment and disappearance of the "spines" normally seen on the conidia) observed by Teel may be all or in part due to the inhibition of lipid synthesis by DMSO. However, it is also possible that DMSO causes an alteration in some pathway other than lipid synthesis that, in turn, leads to the inhibition of lipid synthesis. Furthermore, this pathway is probably unique to the conidia in that DMSO appears to cause significant morphological and biochemical changes only in this area.

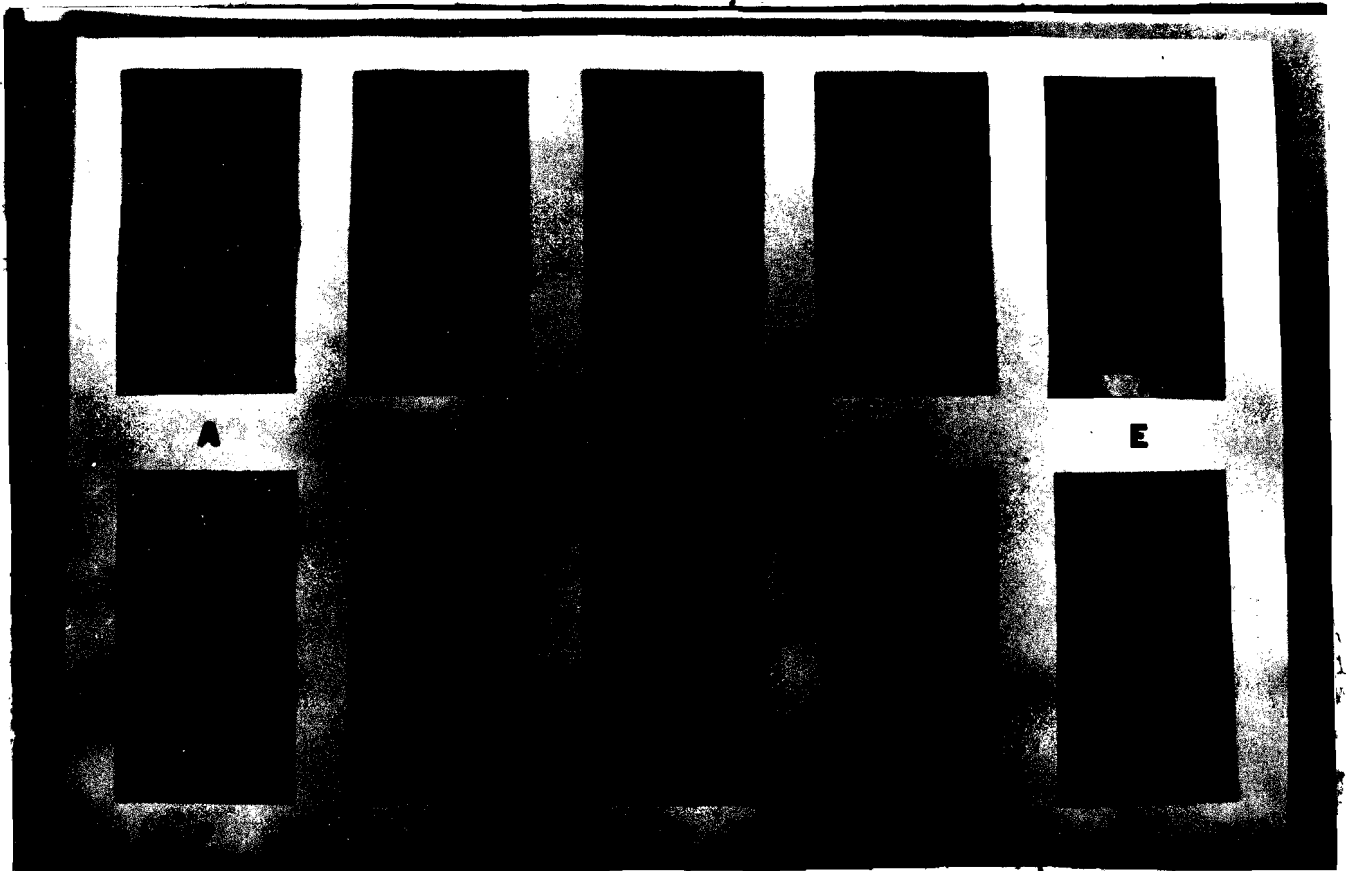


Plate 2. Sudan Black Staining: (A) Conidia from normal medium; (B) Conidia from Sudan Black treated medium; (C) Conidia from Sudan Black treated medium enriched with glucose; (D) Conidia from Sudan Black treated medium enriched with glycerine; (E) Conidia from Sudan Black treated medium enriched with glucose and glycerine. F-J were grown on the same media as A-E except the media were DMSO-amended. Magnification: 400X.

Table 1. Reaction of Conidia and Mycelia to Sudan Black Staining

Flask No.	Ingredients in Medium	Reaction of Conidia	Reaction of Mycelia
1	Agar (Control or normal medium, no additional ingredients)	n <sup>2</sup>	n <sup>2</sup>
2	Agar, DMSO (DMSO-amended control medium)	n <sup>1</sup>	n <sup>2</sup>
3	Agar, Sudan Black B	s <sup>3</sup>	n <sup>2</sup>
4	Agar, Sudan Black B, DMSO	n <sup>1</sup>	n <sup>2</sup>
5	Agar, Sudan Black B, glucose	s <sup>3</sup>	n <sup>2</sup>
6	Agar, Sudan Black B, glucose, DMSO	n <sup>1</sup>	n <sup>2</sup>
7	Agar, Sudan Black B, glycerine	s <sup>3</sup>	n <sup>2</sup>
8	Agar, Sudan Black B, glycerine, DMSO	n <sup>1</sup>	n <sup>2</sup>
9	Agar, Sudan Black B, glucose, glycerine	s <sup>3</sup>	n <sup>2</sup>
10	Agar, Sudan Black B, glucose, glycerine, DMSO	n <sup>1</sup>	n <sup>2</sup>

n - no stain present

s - stain present

<sup>1</sup>natural color bleached

<sup>2</sup>natural color

<sup>3</sup>color more intense than natural color

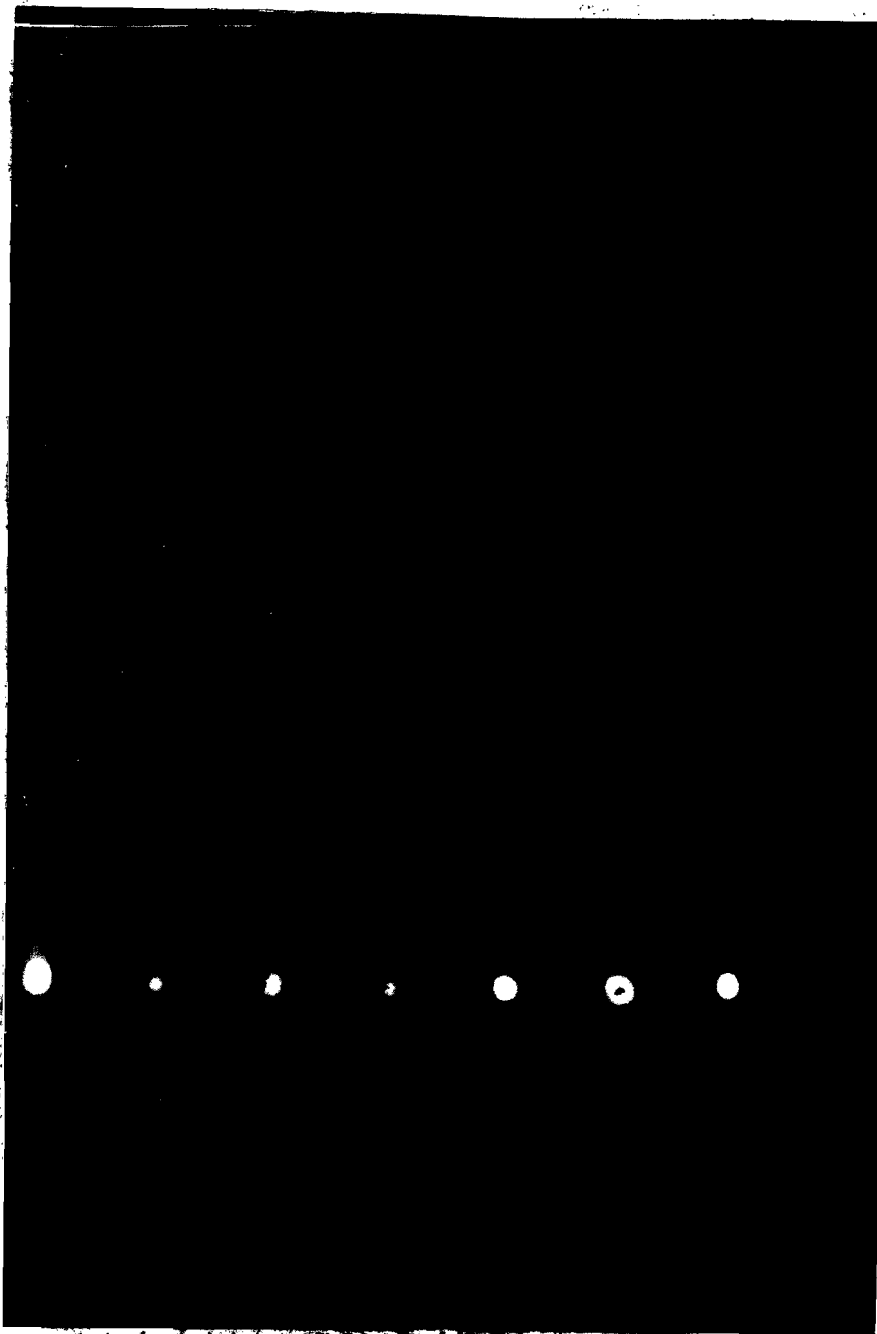


Plate 3. Chromatogram of lipid extracts from conidia.

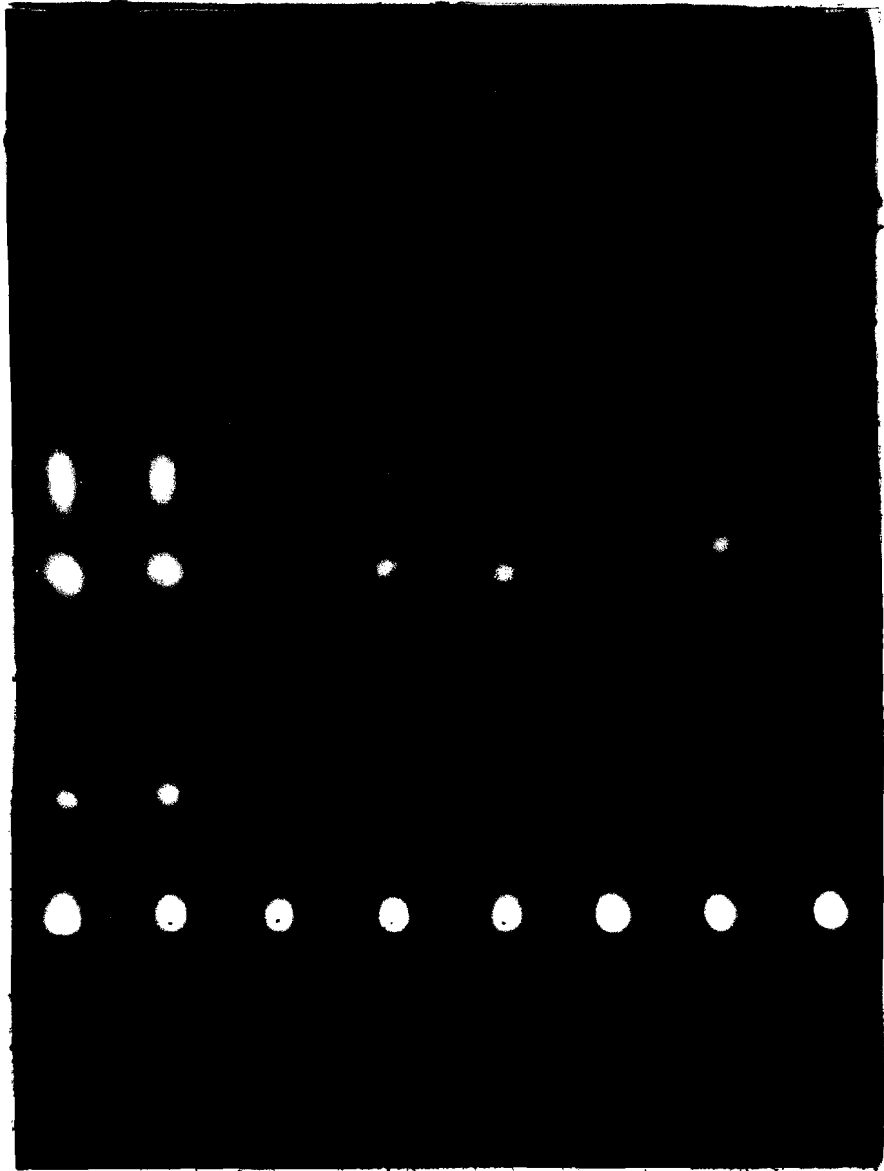


Plate 4. Chromatogram of lipid extracts from mycelial pads.

Table II. Effect of DMSO on Conidia and Mycelia based on Thin-layer Chromatography Analysis

Flask No.	Ingredients in Medium	Reaction of Conidia*	Reaction of Mycelia*
1	Control or normal medium, no additional ingredients	-	-
2	DMSO	+	-
3	glucose	-	-
4	glucose, DMSO	+	-
5	glycerine	-	-
6	glycerine, DMSO	+	-
7	glucose, glycerine	-	-
8	glucose, glycerine, DMSO	+	-

\*grown on agar medium

\*\*grown on broth medium

- spots appeared on chromatogram, lipid synthesis not altered

+ spots failed to appear on chromatogram, lipids altered by DMSO

## SUMMARY

When at 1% concentrations, DMSO inhibits lipid synthesis in the conidia of Aspergillus niger. Sudan Black staining indicates that synthesis of lipids occurs in organisms grown on DMSO-free media. Because the stain is not accumulated in the conidia of the organism when it is grown on DMSO-amended media, it is assumed that lipid synthesis has been disrupted, altered, or inhibited in the presence of the compound.

A comparison of lipid extracts from the conidia and from mycelial pads indicates the inhibition occurs only in the conidia.

In the analysis of lipid extracts of conidia, spots appeared only when the organism was grown on DMSO-free media. This indicates that DMSO causes the inhibition, and this inhibitory action seems specific for the conidia. This correlates with the results obtained from analysis of mycelial pads and Sudan Black staining.

The correlation of inhibition of lipid synthesis, bleaching of the pigment, and disappearance of the "spines" by the same reagent, DMSO, is a phenomenon yet to be elucidated. Further investigation should be pursued to establish whether or not a link exists between these three pathways. When this correlation is established, then perhaps ideas would be forthcoming as to the mechanism by which DMSO inhibits synthesis and how this may or may not effect the morphological changes observed by Teel.

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