THE ROLE OF FUNGAL LIPIDS IN THE HYPERSENSITIVE RESPONSE

by

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We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

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Date September 26, 1967
Studies made on the lipid components of *Microsporum quinckeanum*, have shown that 6 day old fermenter-grown mycelia and that of 21 day old static cultures grown in Sabouraud's glucose liquid medium have a lipid content of approximately 16% by dry weight. Thin layer chromatography showed that the lipid fractions obtained from both methods of cultivation were identical. During the purification of the lipid extracts, a lipoprotein was isolated having 16.45% protein content, and after hydrolysis and chromatography this material demonstrated the presence of eleven amino acids. Dried mycelia, purified lipid and lipoprotein were used separately in complete and incomplete Freund's adjuvant to sensitize guinea pigs and rabbits by various routes. Sensitization of guinea pigs was achieved also by Bloch's method. All animals were challenged by separate intradermal injections of total lipid extracts, lipoprotein and the six lipid fractions. Delayed hypersensitive responses of varying intensities were demonstrated by all sensitized animals. Further investigations were made using lymph nodes removed from rabbits separately sensitized with dermatophyte lipid extracts and lipoprotein. Protein extracts of lymph nodes from these animals were examined electrophoretically. The presence of precipitating antibodies
in sera and lymph node extracts were demonstrated by immuno-
diffusion and precipitin ring tests. Passive transfer tests
were carried out successfully to conclusively demonstrate the
presence of antibodies in the lymph node extracts.
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INTRODUCTION

The immunogenic properties of lipids were discovered in the course of two series of investigation between 1910 and 1920. In one group of experiments Forssman observed that rabbits injected with suspensions of organs obtained from guinea pigs produced antibodies which caused lysis of sheep erythrocytes. The component which was responsible for this Forssman antigen was soluble in alcohol and when injected by itself this material was only weakly antigenic. At about the same time that the above findings were reported, other workers observed that alcoholic extracts of Treponema pallidum, as well as alcoholic extracts of normal organs such as heart served well as sources of the Wasserman antigen used in the complement fixation test for the diagnosis of syphilis. All of these observations then led to the finding of a strongly reacting, organ-specific lipid hapten in brain tissue (3).

Comparatively little is known concerning the immunology of microbial lipids. Also, many conflicting reports have been made concerning their ability to act as antigens (7). However, it is well known that lipids play a role in the virulence of the tubercle bacillus (4), and other observations have shown that lipid materials are associated with the virulence of
certain mycotic pathogens such as *Blastomyces dermatitidis* (10).

Comparatively little work has been done on lipids of dermatophytes and that which has been reported deals mostly with the characterization of the lipid components of the total lipid extract of dermatophytes. Prince (20) has reported on the types of lipid found in a strain of *Trichophyton mentagrophytes*. Audette *et al* (2) presented a quantitative analysis by gas liquid chromatography of fatty acids in *Trichophyton mentagrophytes*. Wirth *et al* (32) reported the presence of brassicasterol and ergosterol in several strains of *Trichophyton rubrum*. Wirth *et al* (31) have also presented a quantitative analysis of the total fatty acids in *Trichophyton rubrum* by gas liquid chromatography. Other workers have been interested in the quantitative studies of lipids in fungi. Al-Doory and Larsh (1), (9) have determined the percentages of acetone-soluble and insoluble lipids contained in 14 species of human pathogenic fungi, 10 of which were dermatophytes. The lipid content was found to range from approximately ten to forty percent, on a dry weight basis. Swanson *et al* (27) showed that the lipid content of *Microsporum quinckeanum* mycelia and spores ranged from approximately 13 to 23 percent. The lipid content can range from a very low amount to a high content depending on the environment. For instance, the type of carbon source available can influence lipid content greatly. When grown on inulin, lipid content was found to be
approximately 5% on a dry weight basis, whereas on glucose, the lipid content was found to be approximately 16%. From the literature review to date, no reference has been made to the possible role of lipids in animal immune responses. Little work has been done in this area of investigation. Tomomatsu (29) prepared somatic dermatophyte antigens by two methods, and then separated cell constituents into fractions termed as crude polysaccharide, crude protein, and crude lipid. These three fractions, as well as the watery extract were tested in rabbits previously infected with *Trichophyton mentagrophytes* var *asteroides* by Bloch's method. As a result of these tests, he demonstrated a positive hypersensitive reaction to the crude lipid extract. Later (30), he again showed a slight skin test reaction with lipid used as antigen intradermally injected into a previously infected animal. Recent experiments on brain lipids have shown that they can also act as haptens (18).

It is known that dermatophytes are composed of a comparatively large lipid content, and that most superficial dermatomycoses produce cutaneous hypersensitivity. Therefore, the purpose of this investigation was to isolate, purify and fractionate the lipid of *Microsporum quinckeaneum*, and to investigate these constituents for their possible role in the hypersensitive response.
MATERIALS AND METHODS

STOCK CULTURES

Subcultures of *Microsporum quinckeaeum*, strain #8 (referred as M.Q8 hereafter) and *Microsporum gypseum* (from F. Blank, Department of Dermatology and Microbiology, Temple University, Philadelphia, Pa.) were maintained on 4\% Sabouraud's glucose agar in Roux flasks grown at 25° for seven days. Subcultures could then be stored up to a period of three months under refrigeration at 4\°.

Inoculum

Microconidia were harvested by shaking the Roux flasks containing 20 ml of sterile 0.85\% saline and approximately 10 gm of sterile glass beads of 5mm diameter.

The microconidia were prepared as an inoculum by filtration through a sterile, coarse, sintered glass funnel (300 ml), observing aseptic techniques. Following filtration, the microconidia were washed three times by centrifugation (12,000 x g for 15 min.) using sterile 0.85\% saline and observing aseptic techniques. Following the washing procedure, the microconidia were resuspended in sterile 0.85\% saline to give an absorbance of .840 at 610 μm (Spectronic 20, 1.2 cm light path).
Medium

Sabouraud's glucose medium

Glucose..........................40 gm
Neopeptone (Difco).............10 gm
Agar (Difco).....................16 gm
Tap Water........................1,000 ml
pH 5.8 --- 6.0 using 1 N HCl. Sterilized by autoclaving at 121°C for 20 minutes.

CULTURES FOR LIPID EXTRACTION

1. Static cultures: Mycelia for the extraction of lipid were grown in 4% Sabouraud's glucose liquid medium in 1000 ml flasks containing 150 ml of medium. One ml of the microconidial suspension per 100 ml of medium was used as inoculum. These cultures were incubated at 25°C for 21 days.

2. Fermenter Cultures: Mycelia for lipid extraction were also grown in a Virtis laboratory fermenter. A 12 liter fermenter jar containing 10 liters of Sabouraud's 4% glucose liquid medium was employed for each batch. One ml of microconidia, optical density of .840 at 610 mp, per 100 ml of medium was used as inoculum. The cultures were grown for 6 days at 25°C with constant aeration and agitation. Agitation was maintained at 250 rpm on the fermenter rheostat. Tributyl citrate was used as the foam control agent applied automatically as required.
Harvesting of Mycelia

Mycelia from both static cultures and fermenter cultures were separately harvested on a Buchner funnel using Whatman #41 filter paper. After separation from the medium, the mycelial mass was washed separately with large amounts of distilled water. Following this step, the mycelial mat was semi-dried by vacuum filtration. The semi-dried mycelia were weighed and a portion was employed for dry weight determination of the total mycelial mass.

Dry Weight Determination

Wet mycelial elements were put into pre-dried (110°C for 12 hrs.), tared aluminum pans. The pans and mycelia were then heated at 110°C for 2-3 hrs., transferred to evacuated desiccators containing CaCl₂, where they were stored for 24 hrs. at room temperature. The pans containing the dried mycelia were then reweighed.

Extraction of Lipids

The mycelial mass was homogenized using a Virtis homogenizer employing chloroform: methanol (2:1 v/v) as the solvent. About 75 mls of solvent were used per 100 gm of mycelial mass. The solvent was then separated from the homogenate using a Buchner funnel and Whatman #41 filter paper. The solvent volume was measured and made up to 250 ml. The solvent and mycelial mass were then put into a Soxhlet extractor whereby the lipids were
extracted using the method of Al-Doory and Larsh (1) with one modification. Instead of using 36 hr. extractions at 65°C, 24 hr. extractions were found to yield the same results and were therefore employed. Prior to purification contaminants such as amino acids, carbohydrates, and glucosamines were detected by thin layer chromatography.

**Purification of Extracted Lipids**

The lipid extract was purified by the "washing technique" of Folch, Lees and Stanley (12). The method involves thorough mixing of the lipid extract with 0.2 (v/v) of its volume of water to which different mineral salts may be added. A biphasic system is obtained. The upper phase contains all the non-lipid substances and the lipid is contained in the lower phase. The washed lipid was concentrated by removing the solvent by flash evaporation and then resuspending the lipid extract in a reduced volume of chloroform: methanol (2:1 v/v).

**Thin Layer Chromatography**

1. **Preparation of plates**

   Silica gel G (According to Stahl) with CaSO_4 was pre-washed with chloroform: methanol (1:1 v/v) employing the method of Thompson and Kies (28). 35 gm of the pre-washed silica gel were slurried with 70 ml of distilled water and transferred to a Camag applicator. A silica gel layer 0.3 mm thick was used. The plates (20 x 20 cm) prepared in the usual manner (21), were allowed to
dry at room temperature for several hours (or overnight) and then
dried in an oven at 110°-115°C for approximately 0.5 hr. and
cooled. They were then stored in a desiccator over CaCl₂. Before
use the plates were activated at 110°-115°C for 0.5 hr. just prior
to the application of samples. They were allowed to cool and
samples were then applied.

The pre-washing was necessary to remove organic material
present in the silica gel which otherwise formed a wide, dark
band at the solvent front after the detecting reagent was applied.

2. Application of Samples

The samples in chloroform: methanol (2:1 v/v) were
applied with micropipettes 2.5 cm from the bottom edge of the
plates. The amounts of application ranged from approximately
100-150 µg of the total lipid extracted. Spots were dried with
a stream of warm air from a drier.

3. Development of Chromatograms

The chromatographic chambers (21.5 x 20.9 x 6 cm)
were prepared just prior to insertion of plates by placing small
amounts of solvent into the chamber. The chambers were lined on
three sides with Whatman #4 filter paper wetted with the devel­
oping solvent. The plates, which had about 0.5 cm of the
adsorbent layer removed off the bottom edge, were placed in the
chamber and allowed to equilibrate for 10-15 mins. Then the
chamber was opened at one corner and 100 ml of solvent were
slowly poured in. The system employed was a modified solvent of Dittmer and Lester (11) consisting of chloroform, methanol, pyridine, water (38:12 v/v, 2%, 2%) and allowed to move approximately 18-18.5 cm from the bottom of the plate. The plates were allowed to dry in a ventilated chromatography drying oven at 50°C.

4. Detection Reagents

Lipids were detected by spraying the plates with a 10% solution of phosphomolybdic acid in ethanol. The plates were then heated in a drying oven at 120°C. As a comparison, a 50% H₂SO₄ solution was also employed (22).

Amino acids were detected by spraying the plates with a modified ninhydrin reagent containing: (A) 50 ml of a 0.2% anhydrous ethanolic nonhydrin solution, 10 ml of glacial acetic acid, 2.0 ml of 2,4,6-collidine and (B) 1.0% solution of Cu(NO₃)₂·3H₂O in ethanol (23). 50 parts of A were mixed with 3 parts of B just prior to use.

Carbohydrates were detected by spraying the plates with an anisaldehyde sulfuric acid reagent. The reagent was 10 ml of a freshly prepared mixture consisting of 9.0 ml of 95% ethanol, 0.5 ml conc. sulfuric acid and 0.5 ml anisaldehyde. 5.0 drops of glacial acetic acid were also added (24). The plates were then heated at 90°-100°C for 10 mins. As an alternative method, anisidine phthalate was also employed (24).

Hexosamines and N-acetylhexosamines were detected
by spraying the thin layer chromatogram with the following reagents (14), (18).

(a) **Hexosamines**

Reagent (a): 0.5 ml acetylacetone in 50 ml butanol.
Reagent (b): 5.0 ml 50% NaOH in 20 ml ethanol.

Combined 0.5 ml of reagent (b) and 10 ml of reagent (a). After spraying, the chromatogram was heated at 105°C for 5.0 mins., cooled, then sprayed with the following reagent.

Reagent (c): 1.0% dimethylaminobenzaldehyde in 9.0 volumes acetone and 1.0 volume conc. HCl.

(b) **N-acetylhexosamines**

The chromatogram was sprayed with reagent (b) above, heated at 105°C for 5 mins., cooled then sprayed with reagent (c).

5. **Tests for purity after "washing"**

After washing by the method of Polch et al (12) the lipid extract was checked for the contaminants as outlined in the previous section except that standards were now employed. The following standards and amounts were spotted on the chromatogram in conjunction with the lipid extract.

A. Amino acids - 0.05 M, 2.0 μl applied.

- Tryptophane
- Alanine
- Phenylalanine

B. Lipids - applied approximately 20 μg.
Oleic  
Stearic  
Lecithin  
C. Carbohydrates - applied 0.5 µg.  
Lactose  
Glucose  
Sucrose  
D. Hexosamine - applied 50 µg.  
E. N-acetylhexosamine - applied 50 µg.  

Fractionation of Lipid Extract

The lipid extract was fractionated by thin layer chromatography. 20 x 20 cm plates were employed and these were prepared as previously outlined. Application of the material was modified from the previous procedure in that the extract was applied as a narrow strip approximately 2.0 cm from the bottom of the plate. The plates were developed in the chambers using the solvent system already outlined. Following drying, the plates were covered with aluminum foil except for a one-half inch strip on either edge of the plate. The unprotected strips of the plates were then sprayed with phosphomolybdic acid in ethanol as previously outlined. Horizontal lines were drawn on the plate separating the six fractions. The sprayed areas were scraped off and discarded while the protected areas were scraped off separately and each fraction placed into a separate container. The lipid
was eluted from the silica gel with chloroform: methanol (2:1), by mixing the slurry with gentle shaking of a partially stoppered flask. The slurry was centrifuged at 10,000 x g for 5 mins. at 4°C. The supernate was poured off into sterile pre-weighed vials. The silica gel was washed twice more, all supernates of each fraction pooled, and the six separate fractions were obtained. The solvent was driven off using a hot stream of air from a chromatography dryer and the vials then placed over CaCl₂ in an evacuated desiccator overnight. The vials were then re-weighed with their lipid contents. Marcol GX mineral oil was now added to each fraction so that the concentration of lipid in each vial was brought to 10 mg/ml. The lipid and mineral oil were now made into a homogeneous suspension using a Vortex mixer.

**Separation of Lipoprotein**

The lipid extract of mycelia obtained from chloroform: methanol resulted in a biphasic system. During washing, by the method of Polch et al (12), the top layer was washed separately and then all the washed fractions were pooled. Upon reducing the volume by flash evaporating to about 1/20th of original volume, it was observed that a residue formed which was now insoluble in chloroform: methanol (2:1), but was soluble in distilled water.

**Identification of Lipoprotein**

Hydrolysis of the water-soluble complex was carried out
using hydrolysis vials containing 1.0 ml of the complex and 2.5 ml of 6N HCl. The vials were sealed after evacuation of air by vacuum pump and hydrolysis carried out at 100°C for 4-6 hours.

Following hydrolysis, the contents of the vial were removed to small test tubes and the acid removed by a stream of air while the tubes were immersed in a hot (90°-95°C) water bath. The residue was washed 3-5 times, or as necessary to remove the HCl, and each time volume was reduced by a stream of air over the hot water bath. After the final washing, the residue was redissolved in 0.5 ml of distilled water and centrifuged to remove any particulate matter.

Subsequent to hydrolysis, paper chromatography was used in the identification of the amino acids. Twenty amino acid standards were employed. Chromatography was carried out on Whatman #4 (18 x 22 inch) paper using single and two dimensional chromatography according to the methods outlined by Ivor Smith (26). For single dimensional chromatography 2 mg/ml standards were employed. 10 μl of the standard amino acids were applied while 50 μl of the hydrolysate was applied. For one-way chromatography the following solvents were employed: n-butanol-acetic acid-water (60:15:25 v/v) and n-butanol-pyridine-water (60:60:60 v/v). The solvent systems for two dimensional chromatography were: n-butanol-acetic acid-water
(60:15:25 v/v) in the first direction, followed by phenol–ammonia (200:1) (26). Detection of the amino acids was carried out by the ninhydrin, collidine reagent previously outlined (see TLC, Detection reagents).

**Protein Determination in Lipoprotein**

Protein content in the lipoprotein was determined employing the method of Lowry et al (17) and using crystalline bovine albumin as a standard protein.

**Detection of Phosphate in Lipoprotein**

The presence of phosphate in the lipid extracts and lipoprotein complex was detected by spraying a thin layer chromatogram with the following mixture: 1 gm ammonium molybdate dissolved in 8 ml H₂O, 3 ml 60% perchloric acid, 3 ml conc. HCl.

The above was made up to 100 ml with acetone. The mixture was sprayed over the chromatogram, dried and the chromatogram placed under UV. The presence of blue fluorescent spots indicated the presence of phosphate (26).

**Determination of Phospholipid in Lipid Extract**

Phospholipid content in the lipid extract was determined by the method of Al-Doory (1) which involved treatment of lipid residue with cold acetone. Since phospholipids are insoluble in acetone, the amount can be calculated by weighing the acetone insoluble precipitate.

**Preparation of Lipid Extract for Immunological Studies**
The lipid extract was prepared by adding 6.0 mls of Marcol-GX mineral oil to 6.892 gms of lipid extract. The purpose was to reduce its viscosity for subsequent handling and preparation as antigenic material.

Preparation of Lipid Fractions for Immunological Studies

The six lipid fractions were suspended in Marcol-GX mineral oil to give a concentration of approximately 10 mg/ml.

Sensitization of Guinea Pigs

The albino guinea pigs used in these series of experiments were 250-300 gm in weight. Sensitization of the guinea pigs was carried out by two methods.

(i) Bloch's method - sensitization by infection (25). The method involves scarifying the flank of an animal with coarse sandpaper and then applying a suspension of spores and mycelia in honey. Several days are required for initiation of infection with the peak being at about the eleventh day. After this the reaction becomes less vivid indicating convalescence. A total of 4-5 weeks therefore is necessary to insure that the animals have fully developed the hypersensitive state.

(ii) Subcutaneous injection of 1.0 ml of mycelial-adjuvant suspension in the scapular region. Because of the lipid nature of the material, diffusion is slow, and therefore 5 weeks were allowed before challenge in order to make sure that the hypersensitive state was obtained.
Preparation of Spores for Bloch's Method

Cultures were grown in Roux flasks on Sabouraud's glucose agar as previously outlined and the spores were harvested as previously reported. The suspension of spores, hyphal elements and saline was centrifuged at 5000 x g, the supernate removed and the pellet added to a small amount of honey. This suspension was then warmed to reduce viscosity and mixed thoroughly employing a glass rod. The same procedure was followed for *M. quinckeaneum* #8 and *M. gypseum*.

Preparation of Mycelia for Sensitization

Cultures were grown in the usual manner in flasks of 4% Sabouraud's glucose liquid medium (previously outlined). The 21 day old mycelia were harvested aseptically, washed repeatedly with distilled sterile water, pre-dried by vacuum filtration and then left to dry in an evacuated desiccator over anhydrous CaCl₂. The dried mycelia then were transferred to a sterile mortar, and observing aseptic techniques, the mycelia were ground to a fine powder. The ground product was then weighed and a known amount was used for suspension with adjuvant.

50 mg of powdered mycelia (approximately 1.92 mg N/ml) were suspended per 1.0 ml of Freund's incomplete adjuvant (Difco). This procedure was repeated for mycelia of *M. gypseum*. 
Guinea Pig Series - Routes of Sensitization and Materials

The guinea pig series involved 7 groups of 6 animals each, and were sensitized according to the following schedule:

Group #1: Spores of M.Q.8 - method of Bruno Bloch.
"#3: As group #1 with M.Q.8 lipid added.
"#4: As group #2 with M.Q.8 lipid added.
"#5: Powdered mycelia of M.Q.8 in Freund's incomplete adjuvant, subcutaneous injection route.
"#6: Powdered mycelia of M. gypseum in Freund's incomplete adjuvant, subcutaneous injection route.
"#7: Controls.

Groups #5 and #6 received 1.0 ml of the suspension in the scapular region once weekly for a total of three weeks.

Hypersensitivity Testing - Guinea Pig Series

Preparation of Animals

Five weeks following the last sensitizing injection the animals were prepared for hypersensitivity testing by clipping the hair on the backs of the animals, and complete removal of hair was completed by the use of a depilating paste (Nair) the day prior to challenge. Then the animals were challenged by intracutaneous injection of 0.1 ml of the following: lipid extract from M.Q.8, the lipid fractions and Marcol-GX mineral oil as a control.
The depililated area of the animals back was divided into 10 equal regions employing a felt-tipped marking pen. The scheme of challenge is shown in Fig. 1.

![Challenge scheme for guinea pig series: A-F: Six lipid fractions. X: Marcol-GX mineral oil used for suspending lipid and lipid fractions.](image)

Observations and recording of observations were carried out at 1/2, 1, 2, 4, 6, 18, 24, 36, 48 and 72 hours after challenge injections.

**Sensitization of Rabbits**

Rabbits used for this series of experiments were the New Zealand White strain with the animals weighing from 2-3 kg. The series included 8 separate groups of 4 animals each. Sensitization was carried out by injecting 1.0 ml of suspension in 0.2 ml quantities subcutaneously at five locations. Inoculations were repeated three times at one week intervals. The sensitizing material employed for each group was the following:
Group 1: 3.0 ml Freund's incomplete adjuvant (Difco), 2.0 ml lipid suspension and 1.0 ml of sterile saline.

Group II: 4.0 ml Freund's incomplete adjuvant, 2.0 ml lipid and 4.0 ml lipoprotein.

Group III: 10.0 ml Freund's incomplete adjuvant, 500 mg fine ground mycelia and 2.0 ml lipid suspension.

Group IV: 10.0 ml Freund's incomplete adjuvant and 10.0 ml of lipoprotein.

Group V: 60 mg crystalline bovine serum albumin, 60 mg (approx.) of lipid extract, 6.0 ml of sterile physiological saline and 6.0 ml Freund's incomplete adjuvant. Material allowed to equilibrate for three days prior to use in sensitization of animals.

In all cases where adjuvant was included in the sensitizing material thorough homogenization of the suspensions was carried out to insure an even suspension of the antigenic material.

Group VI: This group was sensitized by lipoprotein which had been sterilized by millipore filtration using 0.3 μm porosity membrane. Sensitization was carried out by starting with intravenous injections of the marginal vein and later continuing sensitization by intraperitoneal injections according to the following schedule.
Day 1: 0.2 ml, ear vein
4: 0.4 ml, ear vein
7: 0.4 ml, ear vein
10: 0.5 ml, ear vein
14: 1.0 ml, ear vein
21: 1.5 ml, intraperitoneal
28: 1.5 ml,

Group VII: 3.0 ml Freund's complete adjuvant, 2.0 ml lipid suspension and 1.0 ml sterile physiological saline. Sensitization same as groups I to V.

Group VIII: These animals were control animals and therefore were not sensitized.

Hypersensitivity Testing - Rabbit Series

Preparation of Animals

Five weeks following the completion of sensitization the animals were prepared for hypersensitivity testing in the same manner that was used for guinea pig depilation.

The animals were challenged by 0.1 ml intracutaneous injections of the following materials: total lipid extract, six separate lipid components, lipoprotein, Marcol-GX mineral oil, and in Groups IV to VIII a lipid-bovine albumin-Marcol-GX emulsion was also added. The challenge scheme for the rabbit series is shown in Fig. 2.
Fig. 2: Scheme used for challenging the rabbit series:
A-F: denotes lipid fractions.
X: denotes Marcol-GX mineral oil.

Observations and recording of results were carried out at ½, 1, 2, 4, 6, 8, 18, 20, 24, 36, 48 and 72 hours after the challenging injections were completed.

Preparation of Antisera

The rabbits were bled 4½ weeks after the last sensitization. The blood was allowed to clot in the cold and the serum was removed, centrifuged to remove any remaining cells and frozen until used. Groups of animals chosen for this purpose were from Groups II, IV, V, VI, VII, and VIII as previously outlined.

Use of DMSO (dimethylsulfoxide) to Solubilize Lipid Extract

The lipid extract, purified and suspended in mineral oil as previously outlined was added to a minimal volume of a 1/10 dilution of DMSO. This suspension was then used as antigenic
material in the gel diffusion technique for the detection of precipitating antibodies.

**Preparation of Lymph Node Extracts**

Sensitized and non-sensitized rabbits were sacrificed and popliteal and inguinal lymph nodes were excised using aseptic techniques. The excised nodes were removed to sterile petri dishes which were then placed on ice and left for approximately one hour. After cooling, the nodes were firm, while fatty and connective tissue remained soft. Working at ice-bath temperatures, the nodes were removed from the connective tissue. The nodes were then homogenized with minimal distilled water for two minutes. The homogenate was then centrifuged at 4°C at 18,000 x g for 30 minutes. The supernate was removed and the pellet was again homogenized in the same manner. The supernates were pooled and centrifuged at 4°C, 18,000 x g for one hour in cellulose nitrate tubes. During this step, the lipid present in the homogenate concentrated as a solid mass at the surface. A sterile guage 18 syringe needle with cutdown bevel was inserted in the bottom of the centrifuge tube and the contents were drained into another sterile tube, leaving the lipid material in the original centrifuge tube.

These aqueous extracts were reduced in volume by placing them in dialysis tubing at 4°C and allowing streams of air to blow over their surface from a fan. When only a few mls remained,
the contents were emptied into sterile tubes and the volume made up to 10.0 ml using sterile .85% saline. The procedure was repeated for a total of 6 animals, 1 from each group chosen. The protein content of each extract was now determined using the method of Lowry et al (17). The animals sacrificed here were the ones which had previously been immunized and bled for the preparation of antisera.

**Qualitative Precipitation in Gel**

Rabbit antisera and lymph node extracts were screened separately for precipitating antibodies against lipid and lipoprotein by the immunodiffusion method of Ouchterlony (5) using 0.85% Ionagar No. 2 (Colab Laboratories, Inc., Chicago Heights, Ill.) dissolved in sterile borate-saline solution with merthiolate added (1:10,000). The plates were prepared according to a pattern comprising 6 equidistant circumferential wells and 1 central well in the agar, using penicylinders. When the plates had set 0.2 ml of lipoprotein (or lipid) was placed in the centre well, whereas the circumferential wells were prepared by introducing 0.2 mls of lymph node extract (or antisera) from the six different groups of rabbits used. Diffusion was allowed to take place at 30°C for 4-10 days in a humidifier.

**Ring or Interfacial Precipitin Test**

Rabbit antisera and lymph node extracts were examined for precipitating antibodies against lipid and lipoprotein by the use
of the ring or interfacial test according to the method of Campbell et al (6). Test tubes used for the test were 6 x 50 mm. The lymph node extracts for this test were diluted 1:1 with borate saline solution (95 parts .85% saline and 5 parts borate buffer, pH 8.4). Lipoprotein extract was used undiluted. The lipoprotein (.3 ml) was overlaid onto the lymph node extracts (0.5 ml). Observations were made at regular 10 minute intervals for 90 minutes after which time periods were increased to 20 minutes for another two hours. All negative tubes were observed for periods of 18 hours before discarding. Mixtures showing negative results were tested again using different dilutions of lipoprotein (or lipid) and lymph node extracts (or antisera).

Electrophoresis of Lymph Node Extracts

Electrophoresis was carried out on lymph node extracts employing the Gelman Electrophoresis apparatus (Rapid electrophoresis chamber #51101) and Sephaphore 111, a cellulose polyacetate support membrane (Gelman Instrument Co., Ann Arbor, Michigan). The method employed was that outlined in Manual #70176-A (Rapid Electrophoresis, Gelman Instrument Co.) "Procedure for Serum Protein Electrophoresis". The amount of each sample spotted on the cellulose polyacetate strips was 10 µl. The buffer used was Gelman HR buffer. Later a buffer composed of 0.1 M sodium diethyl barbiturate and 0.02 M diethyl barbituric
acid at pH 8.6 was employed (16).

**Passive Sensitization Transfer Test**

Eight guinea pigs were employed for this test. The backs of the animals were depililated by the method previously described and then divided into 12 zones using a felt-tipped marker. In the six areas on the right side of the animal 0.1 ml of antisera was injected intradermally. On the left side of the animal, 0.1 ml of lymph node extract (the sera and the lymph node extract were from the same animal) was injected intradermally. See Fig. 3.

![Diagram of Passive Sensitization Transfer Test](image)

Fig. 3: Scheme for the Passive Sensitization Transfer Test. A-F denotes antisera from 6 different rabbits. A'-F' denotes lymph node extracts corresponding to the antisera.

Five hours later, the animals were injected intradermally with 0.1 ml of lipid or lipoprotein near the previous injection sites where antisera and lymph node extracts were
introduced. Four of the eight animals were challenged with lipid extract and the other four were challenged with lipoprotein. Observations were carried out at 15 min. intervals for the first 2 hours and then at 30 min. intervals for another 6 hours.

RESULTS AND DISCUSSION

Lipid Extraction and Purification

Extraction of lipid from the mycelia of Microsporum quinckeianum, strain #8, grown under static and submerged fermentation conditions revealed, after purification, that the total lipid content of the mycelia was 16% on a dry weight basis regardless of growth techniques when cultivated in the same medium. This value is well within the range of values reported, by various workers, for dermatophytes and other fungi. Values from 1.0 to 40.0% have been reported (1), (9). Some variation in lipid content was expected, since this could arise as a result of different methods of extraction. It was assumed that the procedure used would produce maximum extraction, as there is no assurance that any one method will extract lipid completely from fungal mycelia.

Tests carried out on the gross lipid extract for amino acids, carbohydrates and hexosamines revealed that these were present in relatively large amounts. However, when the lipid extract was purified, no detectable amounts of these "common"
contaminants were observed. Thin layer chromatography was employed as a test for purity, since this was the method of choice, due to the solubility problem of lipids. The use of standards revealed that this method was valid as a means of testing for purity of the lipid extract. Chemical assays for the detection of protein or carbohydrate could not be used owing to the lack of solubility of lipids in aqueous reagents, and in assays where acids were involved, such as the Anthrone reaction, charring of the lipid occurred.

Thin layer chromatography of the purified lipid extracts from both static and submerged fermentation cultures revealed that six fraction were present, and that these were identical under both conditions of mycelial growth when using the same medium (Fig. 4).

The presence of phosphate was detected in Fraction A, and it was shown to be a phospholipid since the spot fluoresced under ultraviolet light. When known lipids were used on TLC, the Rf of Fraction A and lecithin were almost identical. It was therefore assumed that Fraction A could be a lecithin or a lecithin-like material.

When the six lipid fractions were separated by the use of TLC and subsequently eluted, tests for the presence of contaminants showed no detectable amino acids, carbohydrates or hexosamines to be present. It was therefore concluded that
the "washing" procedure employed was effective, and that the separation on TLC of the purified lipid provided an additional step in its purification. Subsequently, TLC and elution were employed as a means of obtaining the six lipid fractions in separate and purified amounts large enough for further experimentation.

The "washing" procedure used yielded a compound which was identified as a lipoprotein complex by demonstrating the presence of proteinaceous material, lipid and phosphate. According to Hanahan (13), lipoprotein possesses these constituents in various concentrations, the phosphate most likely a phospholipid. Subsequent protein determinations demonstrated a protein content of 16.45% on a dry weight basis. The use of the phosphate detecting spray revealed the presence of a small amount of phosphate which was most likely a phospholipid. TLC and a detecting spray for lipid also revealed the presence of lipid material.

Acid hydrolysis of the lipoprotein followed by paper chromatography (Fig. 5, single and two dimensional using standard amino acids) and several different solvent systems showed the presence of the following eleven amino acids: cysteine, cystine, aspartic, glutamic, serine, glycine, threonine, histidine, lysine, arginine and proline. Cystine appeared in very low concentrations while proline appeared in
Fig. 4: Thin layer chromatograph of M.Q.#8 Lipid. Left: static culture; right: submerged, fermentation culture. Solvent: chloroform: methanol (38:12 v/v) 2% pyridine, 2% distilled water. (X\(\frac{1}{2}\) of original).
Fig. 5: Amino acids of M.Q. #8 lipoprotein as demonstrated by paper chromatography.
comparatively large concentrations. Identification of the amino acids was aided by the use of standards in conjunction with the test material and a detecting spray which results in each amino acid having its own characteristic color.

**Skin Test Reactions**

Antigenicity of the six lipid fractions, the total lipid, and the lipoprotein was demonstrated by means of skin test observations carried out on sensitized guinea pigs and rabbits. Guineas pigs were sensitized with both *Microsporum quinckeaneum* strain #8 (M.Q.8) and *Microsporum gypseum*. In two series of animals, extra M.Q.8 lipid was added to the spore-honey suspension for Bloch's method (25) of sensitization to determine if its presence would provide an aggravated inflammatory response in the infectious process. Sensitization procedures and the results for the guinea pig series are outlined in Table 1. Results revealed that a hypersensitive reaction occurred in all cases where animals were sensitized by some method, while the controls showed no reaction. The challenge material in all cases was the six individual lipid fractions, the total lipid extract and the lipoprotein fraction (Table 1). Regardless of the sensitizing agent, whether it was an active infection or whether it was ground mycelia in adjuvant, upon skin test challenge the reactions obtained were of a delayed hypersensitive response, and all sensitized animals reacted in
## Table 1

Hypersensitivity Reactions as Demonstrated by Guinea Pig Skin Tests

<table>
<thead>
<tr>
<th>GROUP AND NO. USED</th>
<th>SENSITIZING MATERIAL AND ROUTE OF SENSITIZATION</th>
<th>CHALLENGING MATERIAL</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (6)</td>
<td>Spores of M.Q.8, method of Bruno Bloch</td>
<td>M.Q.8 lipid components (6) Total lipid, lipoprotein</td>
<td>Positive, necrosis to lipid and lipoprotein</td>
</tr>
<tr>
<td>2 (6)</td>
<td>Spores of M. gypseum, method of Bruno Bloch</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>3 (6)</td>
<td>As group 1 with M.Q.8 lipid added</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>4 (6)</td>
<td>As group 2 with M.Q.8 lipid added</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>5 (6)</td>
<td>Powdered mycelia of M.Q.8 in Freund's, Subcutaneous</td>
<td>As above</td>
<td>Positive for all components</td>
</tr>
<tr>
<td>6 (6)</td>
<td>Powdered mycelia of M. gypseum in Freund's, Subcutaneous</td>
<td>As above</td>
<td>As for group 5</td>
</tr>
<tr>
<td>7 (6)</td>
<td>CONTROLS, no sensitization</td>
<td>As above</td>
<td>Negative</td>
</tr>
</tbody>
</table>
a similar manner and to a similar degree of reactivity. It becomes obvious from the results obtained that the lipid and lipoprotein participate in the induction of a hypersensitive state. The addition of total, purified lipid to the spore suspension did not appear to increase the degree of reactivity as had been anticipated if the lipid had been toxic or possessed the ability to enhance degree of immunogenic reactivity. Necrosis was observed in areas where the animals were challenged with the total lipid extract and the lipoprotein. This may suggest that either the concentration of the lipid fractions was too low to produce a severe enough reaction or that the necrosis was not due to the lipid but rather to a lipid-protein complex and therefore necrosis was due to the protein moiety.

Figure 6 shows representative results obtained using Bloch's method of sensitization with M.Q.8, and therefore displays a homologous skin test reaction.

Fig. 6: Shows representative results obtained using Bloch's method of sensitization with M.Q.8.
Figure 7 shows representative results again using Bloch's method of sensitization, but sensitization was made with a virulent strain of *M. gypseum*. Therefore a lack of immuno-specificity has been demonstrated by the lipid and lipoprotein extracts obtained from two different Microsporum species. Since reports have been made that different fungi possess the same lipids (2) (31) this observation is not unusual that cross-reactivity can occur. This is also in accordance with reports which state that once the allergic state (hypersensitive state) has been created it can be demonstrated by various methods, such as the application of an emulsion of killed fungi, or still more simply by the intracutaneous injection of a crude allergenic extract such as Trichophytin or Microsporin. These observations correspond

![Figure 7](image)

**Fig. 7:** Shows representative results obtained using Bloch's method of sensitization with a virulent strain of *M. gypseum*. For challenge scheme for guinea pig series refer to methods, Fig. 1.
closely to those obtained in the present series of tests. One obtains therefore, inflammatory reactions with the sensitized animal.

Similar results were again shown by animals sensitized with ground mycelia in adjuvant employing *M. Q. 8* and *M. gypseum* (Fig. 8). The control animals showed no reaction upon challenge as demonstrated in Fig. 9.

It is to be noted that in all the results obtained in this series, when skin test challenge of sensitized animals was made with total lipid component and with lipoprotein fraction, a necrotic reaction was obtained. No such reaction was obtained for the six individual, purified lipid fractions. Although no necrosis was present the results were nevertheless positive and indicated either a weaker response or a different type of immunologic reaction than to the total lipid.

![Fig. 8: Results obtained from animals sensitized with ground mycelia in adjuvant employing *M. Q. 8* and *M. gypseum*.](image)
Skin Test Reaction, Rabbit Series

Preliminary observations with the use of rabbits showed that the skin test reactions seemed to provide as sensitive a means of hypersensitivity reaction detection as did guinea pigs. As outlined in Table 11, rabbits were sensitized in groups of four with the complete lipid extract by various routes, and other groups of four by two different routes with the lipoprotein extract. It may be noted that another group of animals was sensitized with a suspension of lipid-bovine albumin which was emulsified and allowed to remain at room temperature for three days prior to use in sensitization. The purpose of this type of sensitization was to determine whether lipid acted as a
### Table II

Hypersensitivity Reactions as Demonstrated by Rabbit Skin Tests

<table>
<thead>
<tr>
<th>GROUP AND NO. USED</th>
<th>SENSITIZING MATERIAL AND ROUTE OF SENSITIZATION</th>
<th>CHALLENGING MATERIAL</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (4)</td>
<td>Lipid from M.Q.8, incomplete Freund's adjuvant; subcutaneous</td>
<td>Lipid components (6), total lipid, lipoprotein, Marcol-GX</td>
<td>Positive, necrosis to lipid and lipid protein</td>
</tr>
<tr>
<td>2 (4)</td>
<td>Lipid from M.Q.8, lipoprotein, incomplete Freund's adjuvant; subcutaneous</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>3 (4)</td>
<td>Lipid from M.Q.8, dried mycelia from M.Q.8, incomplete Freund's adjuvant; subcutaneous</td>
<td>As above</td>
<td>As above</td>
</tr>
<tr>
<td>4 (4)</td>
<td>Lipoprotein and Freund's adjuvant; subcutaneous</td>
<td>As above plus a lipid-bovine albumin-Marcol-GX emulsion</td>
<td>Positive, necrosis to lipoprotein</td>
</tr>
<tr>
<td>5 (4)</td>
<td>Lipid, bovine albumin and incomplete Freund's adjuvant; subcutaneous</td>
<td>As in Group 4</td>
<td>Positive, necrosis to lipid-albumin emulsion</td>
</tr>
<tr>
<td>6 (4)</td>
<td>Lipoprotein, intravenously and intraperitoneally</td>
<td>As in Group 4</td>
<td>Positive, light necrosis to lipoprotein</td>
</tr>
<tr>
<td>7 (4)</td>
<td>Lipid and Freund's adjuvant; subcutaneous</td>
<td>As in Group 4</td>
<td>Positive, necrosis to lipid, lipoprotein, lipid albumin emulsion</td>
</tr>
<tr>
<td>8 (4)</td>
<td>CONTROLS, no sensitization</td>
<td>As in Group 4</td>
<td>No reactions</td>
</tr>
</tbody>
</table>
better antigen in presence of a carrier protein. Niedieck et al (18), while working with myelin lipid hapten, reported that in the presence of carrier protein (bovine serum albumin) myelin cerebroside produced antibodies in rabbits which would precipitate with cerebroside-cholesterol-lecithin emulsions. Animals which did not receive injections with carrier protein but cholesterol alone did not produce antibodies which would precipitate with this antigenic complex. Their results indicate that cerebroside is a hapten according to the definition given by Landsteiner (8).

Fig. 10: Representative results observed in rabbit groups 1-4, and groups 6, 7. For challenge scheme in the rabbit series refer to Fig. 2.

Sensitization procedures and the results for the rabbit series are outlined in Table II. Representative results of groups 1 to 4 and of groups 6 and 7, entailing 24 animals can be observed in Fig. 10. All animals in these groups showed a
delayed type of hypersensitive reaction to the six individual lipid components, the lipid and the lipoprotein. However, at about 24 hours after challenge, necrosis was observed at the total lipid and the lipoprotein challenge sites and this progressively increased in intensity until 36 hours, and then slowly diminished. In group 6, however, there was no necrosis to the total lipid as well as the individual lipid fractions. This could be expected, since the animals in this group were sensitized with lipoprotein alone. Since a positive reaction without necrosis was nevertheless obtained it may be assumed that either the lipid component is somewhat common to the total lipid extract or that lipid induced reactions are not specific and that variable cross-reactivity occurs. The necrotizing activity in this case may also be due to an immunologic specific protein moiety.

Fig. 11 shows typical results obtained for group 5 observed at 28 hours after challenge exemplary of all four animals in this group and depicts an increase in necrosis when lipid was added to bovine albumin prior to sensitization.

These results seem to indicate that in the presence of albumin, the lipid may complex with the protein and therefore act as better antigenic material than when used alone. These results support the observations obtained by Niedieck et al (18). Fig. 12 shows typical results of skin test challenge using a non-
sensitized or control rabbit demonstrating absence of immunologic reactivity.

Fig. 11: Results obtained for group 5 observed 28 hours after challenge. Note the increase in necrosis to the lipid-bovine albumin emulsion. Sensitization induced by lipid-bovine albumin emulsion.

Fig. 12: Results obtained when a non-sensitized control animal was challenged in the usual manner.
From the results presented until now, delayed hypersensitivity reactions were obtained with all skin test challenge antigenic materials in sensitized animals. Since the control animals showed no reactions, it was concluded that sensitized animals when challenged displayed a delayed hypersensitive reaction due to the presence of antibodies produced against an antigenic determinant site conferred by the lipid itself, the lipoprotein and/or lipid-serum protein complexes. An immediate hypersensitive response was not demonstrated. Necrosis, however, was obtained at the lipoprotein challenge site.

Since all the immunologic reactions obtained so far appeared to be of the delayed hypersensitive type, antisera was obtained from representative animals of the various groups in an attempt to demonstrate the presence or absence of circulating antibodies. Antisera were obtained from groups 2, 4, 5, 6, 7 and 8 as outlined in Table II. As lipids are insoluble in aqueous solutions, a problem arose when an attempt was made to test antisera for antibodies against the lipid and lipid fractions. The lack of solubility was overcome by the use of dimethyl sulfoxide (DMSO). In an attempt to test for interference from DMSO in gel diffusion reactions, known antigen-antibody systems were treated with DMSO and tested by gel diffusion techniques outlined in methods. The known immunologic systems evaluated were:
Beef serum: Rabbit antibeef serum
Horse serum: Rabbit antihorse serum
Bean plant virus: Rabbit bean plant virus antiserum

The presence of DMSO in these systems did not interfere with the reactions and very clear precipitin lines were obtained. Subsequently rabbit antisera obtained from aforementioned sensitized groups were tested against the total lipid, the lipid fractions, and lipoprotein by the gel diffusion technique. No precipitin lines were obtained by this method even after 10 days in a humidifying chamber. It was therefore concluded at this stage that the system was either not a precipitable system, or the lipid could not act as a precipitating antigen without serum protein, or that there were no circulating antibodies in the antisera. The surprising observation was that there did not appear to be any precipitating antibodies against the lipoprotein, since antisera is sometimes used to test for lipoprotein classes (14). However, since the reaction to lipoprotein was also of the delayed type it was thought that there were no circulating antibodies present.

Since delayed hypersensitive responses were obtained, and since in such responses it has been shown that injection of lymphocytes and other nucleated cells from the blood and lymphoid tissue of a sensitive individual will confer,
passively, the same delayed-type hypersensitivity on the recipient, it was thought that any antibodies that were present were "tissue-fixed" in the lymphoid tissue.

In an attempt to demonstrate either the presence or absence of such "tissue-fixed" antibodies the following groups of rabbits were chosen. Groups 2, 4, 5, 6, 7, and 8 as outlined in Table II.

The lymph node extracts were examined for protein content by the Lowry method (17). The results are shown in Table III.

Table III

Protein content as demonstrated by the Lowry method in the lymph node extracts obtained. One animal per group was used to obtain the extract.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>6.00</td>
</tr>
<tr>
<td>4</td>
<td>3.20</td>
</tr>
<tr>
<td>5</td>
<td>2.25</td>
</tr>
<tr>
<td>6</td>
<td>3.50</td>
</tr>
<tr>
<td>7</td>
<td>5.00</td>
</tr>
<tr>
<td>8</td>
<td>6.60</td>
</tr>
</tbody>
</table>

Popliteal and inguinal lymph node extracts were examined by electrophoretic techniques on cellulose acetate strips and only very slight differences in band formation were observed when compared to the controls. Consequently, the precipitin ring test was chosen in an attempt to elucidate whether antibodies were present in these extracts. Lipoprotein
extracts were overlaid onto solutions of lymph node extracts from the six animals outlined and the results obtained can be seen in Fig. 13 showing strong precipitin reactions, except where the lymph node extract obtained from a non-sensitized animal was employed.

Fig. 13: Results of ring precipitin test when lipoprotein was overlaid onto lymph node extracts. The numbers of the tubes correspond to lymph node extracts of animals sensitized in the following: 1, intraperitoneally with lipoprotein; 2, subcutaneously with lipoprotein in complete Freund's adjuvant; 3, subcutaneously with Freund's incomplete adjuvant with lipid and bovine serum albumin; 4, subcutaneously with Freund's incomplete adjuvant with lipid and lipoprotein; 5, control: non sensitized; 6, not shown, sensitized to lipid contained in complete Freund's adjuvant, subcutaneously.

From the results obtained, it appears that precipitating antibodies were present in the lymphoid tissue against the lipoprotein. A similar test was attempted using lipid and the six lipid fractions, but due to the insolubility of the lipid material no results were obtained. When, in the presence of DMSO,
solubility was achieved but no results were observed. It was thus concluded that either this was not a precipitating system (no carrier animal protein present) or there was interference from the DMSO. A lack of lymphoid-bound antibodies was not considered since all skin test challenges produced very strong reactions in all sensitized animals as previously shown. It was concluded that this system was not applicable to the materials involved.

To further demonstrate the presence of lymphoid contained antibodies against the lipoprotein, the Ouchterlony gel diffusion technique in agar was employed. The center well contained the lipoprotein extract and the circumferential wells contained various sources of lymph node extracts as previously outlined. The results of this test are shown in Fig. 14.

Fig. 14: Ouchterlony gel diffusion employing lymph node extracts in circumferential wells and lipoprotein extract in the center well. Numbers 1-6 refer to the sources of extracts as outlined for Fig. 13.
It appears, therefore, that cell-bound antibodies are present against the lipoprotein in lymph node extracts as demonstrated by the previous tests.

Since a hypersensitive response of the delayed type was observed in all animals sensitized, further experiments were attempted to elucidate the presence of cell-bound antibodies. The passive transfer technique was attempted in which antisera and corresponding lymph node extracts were separately injected intradermally. These sites then were challenged with lipid and lipoprotein (4 animals in each group). The results to these tests can be observed in Figs. 15 and 16.

Fig. 15: Passive transfer test. Rt. side of animal contains intradermal injection of sera corresponding to lymph node extracts as previously outlined. Lt. side contains lymph node extracts as previously outlined. Challenged with lipoprotein.
Fig. 16: Passive transfer test. Rt. side of animal contains intradermal injection of sera corresponding to lymph node extracts as previously outlined. Lt. side contains lymph node extracts injected intradermally as previously outlined. Challenged with lipid.

These results indicate that lymphoid tissue-bound antibodies were present in the lymph node extracts as demonstrated by the passive transfer of antibodies to non-sensitized animals, and that they were present not only to lipoprotein as previously demonstrated by gel diffusion and precipitin ring tests but were also present against the total lipid extract. It is thought therefore that they were not demonstrable against the lipid by other tests, as either the system was not a precipitating one, or carrier proteins are necessary. Since the site of the animal where serum was injected intradermally produced no reaction, it is concluded that the reactions observed are due to passive transfer of lymphoid-bound antibodies and not due to any toxic effects of the lipid or the lipoprotein.
SUMMARY

Under the conditions of the experiments carried out the observations made may be summarized as follows: the lipid content of Microsporum quinckeaneum strain #8 is 16% regardless of growth techniques when the same medium is used. A lipoprotein complex was also present regardless of growth techniques. Whether grown as static culture or as submerged culture in a fermenter, the lipid components were the same as demonstrated by thin layer chromatography. Acid hydrolysis of the lipoprotein revealed the presence of eleven amino acids, namely, cysteine, cystine, aspartic, glutamic, serine, glycine, threonine, histidine, lysine, arginine and proline. Positive skin test reactions were obtained for the lipid extract and for the individual lipid components regardless of the route of sensitization. Lack of immunospecificity for the lipid complex was also demonstrated. The addition of bovine albumin to the lipid complex resulted in a stronger skin test reaction when challenged with the same material. Since non-sensitized control animals demonstrated no reaction upon challenge with the lipid or lipoprotein it appears that the purified lipid does act either as an antigen or as a hapten. Serum protein may act as a carrier of the haptenic moiety. There is a lack of immunospecificity as shown by the results when lipoprotein was used as the antigenic material. Species specific anti-
genicity does not appear to be present in this cellular fraction which has been demonstrated for the first time with this organism. Cell-bound antibodies were present to the lipoprotein as demonstrated by the ring precipitin test, the gel diffusion technique and the passive transfer technique. It is concluded that the same is true for the lipid complex as demonstrated by the results obtained by the skin test reactions, however, because of the lack of solubility of these compounds this could not be demonstrated in any other manner.
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