Observations of Fungal Growth *in vitro* and *in vivo*

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The cultivation of pathogenic fungi from clinical specimens imposes limitations not commonly met in subculturing strains that have already been isolated.

Table 1 records the effect of various media, incubated at two temperatures, on the rate of isolation of three deep-seated pathogenic fungi. The compositions of the media are listed in Table 2. The penicillin-streptomycin blood agar medium (P & S) ([2]) is the most complex organically. It contains 10 per cent blood as one of its components. The choramphenicol agar ([6]) contains a variety of nutrients, such as beef extract, peptone, yeast extract and dextrose. Although it is a nutritionally rich mixture, it lacks the blood and serum factors found in the P & S medium. The medium with the least nutritional fortification is Sabouraud's dextrose agar. It utilizes 1 per cent peptone as its nitrogen source. This medium bears little resemblance to the original one described by Sabouraud ([3]), which employed a peptone and dextrose or maltose of very low purity but obviously of much greater nutritional value.

The rate of isolation of deep-seated fungal pathogens bears a direct relation to the nutritional complexity of the medium. *Histoplasma capsulatum*, which is the most fastidious of the three fungi studied (Table 1) shows the greatest difference in the rate of isolation on the three media.

Both *H. capsulatum* and *Blastomyces dermatitidis* are biphasic organisms growing in the mycelial phase at 25°C and in the yeast-like phase at 37°C. Temperature is one of the prime requisites for this phase change, but the nutritional character of the medium is of considerable importance. The formation of the yeast phase is greatly fa-

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TABLE 1.—*Effectiveness of various media at 25°C and 37°C in the isolation of pathogenic fungi from clinical specimens.*

Organism & numbers isolated	Incubation temperature, °C.	Percentage isolation		
		PAS blood agar	Chloramphenicol agar	Sabouraud's dextrose agar
Blastomyces dermatitidis 46	25	68.8	57.7	18.5
	37	61.5	25.9	. . .
Histoplasma capsulatum 17	25	88.2	47.0	20.0
	37	18.7	0	. . .
Coccidioides immitis 27	25	84.6	88.4	77.7
	37	90.0	90.0	. . .

TABLE 2.—*Composition of media.*

Penicillin–streptomycin blood agar

The agar base is prepared as follows:

Heart infusion agar (Bacto)	40 gm.
Gelatin	1 gm.
Dextrose	2.5 gm.
Distilled water	1000 ml.

To each liter of the melted and cooled agar, the antibiotics dissolved in blood are added:

Blood (human or horse)	100 ml.
Penicillin	50 mg.
Streptomycin	50 mg.

Chloramphenicol agar

		Salt A	
Tryptone	3 gm.	NaH_2PO_4	25 gm.
Beef extract	2 gm.	Na_2HPO_4	25 gm.
Yeast extract (Difco)	5 gm.	H_2O	250 ml.
Dextrose	5 gm.		
Starch (soluble)	2 gm.	Salt C	
Dextrin	1 gm.	$MgSO_4–7H_2O$	10 gm.
Chloramphenicol	125 mg.	$FeSO_4–7H_2O$	0.5 gm.
Salt A	10 ml.	NaCl	0.5 gm.
Salt C	20 ml.	$MnSO_4–7H_2O$	2.0 gm.
Agar	17 gm.	H_2O	250 ml.
Distilled water	970 ml.		

Chloramphenicol is first dissolved in 3 or 4 ml. of alcohol and then added to the boiling medium. The pH is adjusted to 6.7.

Sabouraud's dextrose agar

Peptone	10 gm.
Glucose	40 gm.
Agar	17 gm.
Distilled water	1000 ml.

vored by the presence of blood in the medium. The temperature of incubation is often critical when related to the success of isolation. This is especially conspicuous with *H. capsulatum*, where a very great difference is apparent. In numerous instances cultures incubated at 37°C for two weeks gave no indication of growth whatever, but if the medium was subsequently kept at 25°C, the mycelial phase would appear. The organism was present in the inoculated clinical material and was viable, but could not initiate growth at the higher temperature.

The very poor showing of Sabouraud's dextrose agar is not due entirely to the nutritional aspects of the medium. All media were inoculated with clinical material, and specimens such as sputum, gastric contents, pus, etc., often contained bacteria or other fast-growing saprophytic molds, which overgrew the medium and greatly reduced the chances of any pathogen to compete.

It should be of interest to note that the nutritionally rich media actually exhibited a suppressive effect on the growth of saprophytic molds. They commonly did less well on the complex media than on the simple. Higher temperatures of incubation also reduced the rate and amount of their growth.

While rate of isolation is favored by a nutritionally fortified medium, some morphologic aspects may be slow in forming or completely inhibited. The tuberculate chlamydospore, which is part of the definitive structure of *H. capsulatum* in the mycelial phase, commonly does not form on blood-containing media. The chlamydospore itself forms, but the secondary spores or tubercules do not arise. Transfer to a simpler medium, such as Sabouraud's agar, will produce the typical sporulation.

Coccidioides immitis grows only in the mycelial phase under ordinary culture conditions and is also more consistently recovered from clinical specimens on a variety of media incubated at room and body temperatures. The general impression gained from these studies indicates that the more fastidious an organism appears, the better it grows on primary isolation when complex organic compounds, such as those contained in blood, are present. The superficial fungal pathogens, the dermatophytes, are commonly more readily cultured from clinical specimens than the deep seated fungi. The faviform group is an exception to this observation. These fungi serve as good models to study the effect of growth factors and their antagonists (4).

Table 3 shows the effect of thiamine and neopyrithiamine alone and in combination on the growth of *Microsporum audouini* on Sabouraud's agar after a 30 day incubation period. Neopyrithiamine inhibits at a level of 0.5 μgm./ml. The inhibitory effect can be reversed by the addition of thiamine, but, as the level of the growth factor is reduced, the inhibitory effect of the antagonist again becomes apparent.

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TABLE 3.—*Effect of neopyrithiamine alone or with thiamine on the growth of* Microsporum audouini.

Micrograms per milliliter		Strain 2303[a]	Strain 2146[a]
Neopyrithiamine	Thiamine		
Control		++++	++++
5.0	...	±	±
3.0	...	±	±
1.0	...	±	(contaminated)
0.5	...	+	(contaminated)
3.0	0.30	++++	++++
3.0	0.10	++++	++++
3.0	0.03	++	+++
3.0	0.01	±	++

[a]Two stock strains on Sabouraud's agar, incubated for 30 days.

compounds on the primary isolation of *Microsporum audouini* and *Microsporum canis* from the infected hairs is indicated in Table 4. Strain 2 of *Microsporum audouini* is completely inhibited by 5 μgm. of neopyrithiamine in the medium, but Strain 1 produced some rudimentary filaments, although growth did not continue. Thiamine successfully overcame the inhibitory activity of the antagonist, a fact indicating that a reversible inhibition system had been established. While *Microsporum canis* shows some inhibition, it still exhibits visible growth. These results show that *Microsporum audouini* has an essential requirement for thiamine, i.e., that it cannot synthesize its own needs. *Microsporum canis*, on the other hand, is stimulated by added growth factor, but has the ability to produce sufficient amounts to initiate and maintain growth. It is also apparent that Sabouraud's agar normally contains enough thiamine to serve the needs of *Microsporum audouini* for growth.

In order to test the reversible activity of this pair of compounds more critically, the completely synthetic medium of Henderson and Snell ([1]) was employed. The results are listed in Table 5. Note that a level of neopyrithiamine required to inhibit both audouini and canis strains completely is only 0.5 μgm./ml. It requires 0.05 μgm./ml. of thiamine to overcome the inhibition. The 4+ growth of the controls can be attributed to the carryover of thiamine in the inoculum plus the natural source of the vitamin in Sabouraud's agar.

The reversible inhibition of growth factor and antagonist can be demonstrated only when the organism being tested has an essential requirement for the growth factor. The effect of another pair of an-

TABLE 4.—*The effect of neopyrithiamine and neopyrithiamine plus thiamine on growth of* Microsporum audouini *and* Microsporum canis *from infected hairs.*

Micrograms per milliliter		*Microsporum audouini* 1	*Microsporum audouini* 2	*Microsporum canis*
Neopyrithiamine	Thiamine			
Control		++++	++++	++++
5.0	...	+ Microscopic	0	++
3.0	...	+ Microscopic	+ Microscopic	++
1.0	...	+ Microscopic	+ Microscopic	++
0.5	...	+ Microscopic	+ Microscopic	++++
3.0	0.3	++++	++++	++++
3.0	0.1	++++	++++	++++
3.0	0.03	++++	++++	++++
3.0	0.01	++	++++	++++

TABLE 5.—*Effect of thiamine and neopyrithiamine or both on the growth of microspora.*

Micrograms per milliliter		*Microsporum audouini*[a]		*Microsporum canis*[a] 196
Neopyrithiamine	Thiamine	2145	2192	
Control[b]		++++	++++	++++
0.5		0	0	0
	0.5	++++	++++	++++
0.5	0.01	0	0	0
0.5	0.03	0	0	0
0.5	0.05	++++	++++	++++

[a]Henderson and Snell's medium (17 days of incubation).
[b]Sabouraud's agar.

TABLE 6.—*Effect of Aminopterin and folic acid or both on the growth of several dermatophytes.*

Micrograms per milliliter		*Microsporum audouini*[a] 2303	*Microsporum canis*[a] 196	*Trichophyton mentagrophytes*[a] 167
Aminopterin	Folic acid			
Control[b]		++++	++++	++++
	0.002	++++	++++	++++
	0.0002	++++	++++	++++
2.0		++++	++++	++++
0.2		++++	++++	++++
2.0	0.002	++++	++++	++++
0.2	0.002	++++	++++	++++

[a]Henderson and Snell's Medium (16 days incubation).
[b]Sabouraud's agar.

tagonists for a factor not essential for the growth of dermatophytes is included in Table 6. Folic acid and its antagonist Aminopterin are employed. All three organisms show the same degree of growth in all tubes, a fact indicating that folic acid is not an essential growth factor.

The so-called vitamin growth factors have been studied extensively for a variety of organisms and are well understood. A similar area, however, that has yet to be more fully developed, is the effect of the peptides. Wooley ([7]) has synthesized a peptide, seryl-glycyl-serine, which has activity similar to that of the aqueous liver extract of Sprince and Wooley known as "Streptogenin." Both the known peptide and the liver factor can be inhibited by synthetic peptides containing serine, glycine and aspartic acid. Other imperfectly described substances isolated from a variety of natural sources possess stimulating activity and appear to be peptide in nature. More work needs be done in this area, especially with organisms that are fastidious or difficult to grow.

Another avenue of approach that needs to be exploited for better understanding of the infectious process, is observation of the host-parasite relationship.

The relation between lymphomata and fungous infections has long been noted. There is clinical evidence also that patients most prone to deep seated fungal infections are physiologically abnormal.

A study was instituted to determine if the urinary amino acid excretion patterns of persons with known fungal infections varied from normal patterns. Twenty-four-hour urine specimens were collected and the unbound or "free" amino acid contents were determined for 15 of the acids. The microbiologic assay method of Henderson and Snell ([1]) was employed. The data obtained are compiled in Table 7. The normal values listed are based on data collected from 90 men and women ([5]). As the patterns of the two sexes are essentially the same, the average of the combined values is employed. The second column of figures is twice the standard deviation of the mean of the normal values. Only average values from the infected cases that are above or below the normal value plus or minus twice the standard deviation of the mean are considered to be abnormal. When these criteria are used it is of interest to note that cases of blastomycosis, coccidioidomycosis, and cryptococcus infection give abnormally high excretion values of some of the amino acids, while cases of histoplasmosis show a low excretion pattern.

The amino acids that are abnormal in cases of blastomycosis are glutamic acid, leucine, lysine, methionine, and valine. The pattern for coccidioides infections is aspartic acid, isoleucine, and proline. Cryptococcosis cases show the greatest number of increased excretions, isoleucine, leucine, methionine, phenylalanine, proline, tyrosine, and valine being elevated. Histoplasma infections give consistently low excre-

TABLE 7.—*Urinary amino acid excretion patterns in normal subjects and patients with deep seated fungal infections.*

Amino acid	Normal values[a]	2 X SD[b]	Infecting organism and number of cases			
			Blastomyces 4	Histoplasma 3	Coccidioides 4	Cryptococcus 6
Arginine	21	7.5	28	16	24	25
Aspartic acid	65	24	75	43	92	65
Cystine	94	39	132	56	121	132
Glutamic acid	222	73	319	227	288	221
Histidine	217	97	280	127	240	204
Isoleucine	6.8	2.2	6.9	3.9	12	9
Leucine	21	8.2	32	28	26	33
Lysine	63	29	142	40	75	80
Methionine	3.4	1.3	5.7	2.3	4.6	5.5
Phenylalanine	18	5.2	21	11	19	23
Proline	29	10	37	30	43	41
Threonine	48	19	49	25	65	44
Tryptophane	19	7.4	22	8.8	24	24
Tyrosine	43	13	52	25	51	61
Valine	17	5.2	25	20	22	28

[a]Milligrams excreted in 24 hours.
[b]2 times the Standard Deviation of the mean.

tions for the amino acids involved. These include isoleucine, phenylalanine, threonine, tryptophane, and tyrosine.

The specific patterns of excretion indicate abnormal metabolism of amino acids. It is unlikely that unusual kidney function is the cause of the patterns because all of the amino acid excretions would be abnormal, as they are in Wilson's disease.

The significance of these patterns is still being sought, but they do indicate that persons with deep-seated fungal infections do possess physiologic functions that are not normal.

The discovery of abnormal function in physiologic processes signifies that unusual metabolic products may be produced, which can either stimulate or suppress an invading organism.

It would be of value to fractionate the tissues of normal and infected persons in a search for anomalous substances. These, if found, could then be checked for their effect upon invading pathogens.

SUMMARY

Three areas of endeavor have been discussed, viz.,

1. The effect of various media on the rate of isolation of deep seated fungal pathogens from clinical specimens.
2. The effect of growth factors and their antagonists on the reversible inhibition of growth among the dermatophytes.
3. The relation of fungal infections to abnormal urinary amino acid excretion patterns in infected patients.

Further approaches to the problems of culturing pathogens are discussed.

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DISCUSSION

Dr. Emmons. Dr. Ulrich has reviewed the evidence that a nutritionally enriched medium and an incubation temperature well below the maximum for growth favor the initial isolation of *Histoplasma capsulatum, Blastomyces dermatitidis* and *Coccidioides immitis.* Once isolated, in subculture, these fungi are less fastidious. He has pointed out that the inhibitory effect of neopyrithiamine can be overcome by supplying thiamine to dermatophytes that are deficient for this growth promoting factor. Finally, he has pointed out that, from the viewpoint of host susceptibility, variations from normal metabolism of certain amino acids are found in some persons with systemic mycoses.

In opening the discussion of Dr. Ulrich's interesting paper, I shall direct my comments to his first two points. Temperature of incubation is one of the determining factors for both growth rate and form in some dimorphic fungi. *Histoplasma capsulatum,* which is a mold at ambient temperatures, becomes an intracellular yeast *in vivo.* Its ability to grow and multiply in histiocytes without apparent early damage to the nucleus of the cell suggests a long evolutionary development as a parasite. Yet *H. capsulatum* reverts at once to the mold form on ordinary media at temperatures below 35°C, and it ordinarily leads an independent saprophytic existence in soil. A highly adapted intracellular habitat, in the case of *Histoplasma,* is not associated with host-dependency.

Despite the use of enriched media and incubation over an appropriate range of temperatures, we have not been able to obtain *in vitro* growth of such pathogenic fungi as *Rhinosporidium seeberi* and *Loboa loboi.* Both these fungi are abundant and conspicuous in lesions, but they do not grow *in vitro* under any conditions yet devised. In these respects they are like *Mycobacterium leprae.* However, in the case of these pathogenic fungi, difficulties of *in vitro* propagation are not related to an intracellular habitat. On the contrary *R. seeberi* and *L. loboi* are extracellular. Their special nutritional requirements have not yet been defined. We have no way of estimating the antiquity of their parasitic relationship, but it is generally assumed that like most other fungal pathogens of man, they have an independent existence in man's environment.

Mycologists have been more successful, after many failures, in the isolation of certain small yeast-like fungi found on the skin. *Pityrosporum ovale* is commonly present in great numbers on the human skin, but is difficult to isolate except on special media. In 1939 Benham ([1]) reviewed earlier attempts and reported that she was able to grow and maintain cultures on wort agar overlayed or enriched with any one of several fats and oils. *P. ovale* can be isolated easily from epidermal scales by floating them on the surface of neopeptone-dextrose broth to which glycerol is added ([2]) to a final concentration of 23-44 per cent. After several days' incubation on this medium, most other microorganisms are dead and subcultures of *P. ovale* to a medium overlayed with oil or an ether extract of lanolin are successful.

A second species of *Pityrosporum* (*P. orbiculare*) was described by Gordon ([4]). It is associated with pityriasis versicolor and is perhaps the fungus, *Malassezia furfur,* which is seen so abundantly in the

epidermal scales of this disease, and which for more than a hundred years, was considered noncultivable.

A third species, *P. canis*, is found in the ears and on the skin of dogs and other animals ([5]). It resembles the previous species, but does not require addition of oil for *in vitro* growth.

Mycology presents many anomalous relationships between complexity, adaptation, and ease or difficulty of isolation and culture *in vitro*. Most of the fungi that cause subcutaneous and systemic mycoses in man are normally free-living saprophytes capable of indefinite saprophytic existence in suitable environments. Man becomes a host only by accident ([3]), but the erstwhile saprophytic fungus can become a virulent pathogen. *Cicinnobolus cesatii* is a specialized intracellular parasite of other molds; yet it grows on a variety of media in the laboratory. On the other hand, the rust fungi have complex life cycles, are adapted to parasitism that may require two unrelated species of host plants, and many have not been grown *in vitro*.

The papers and discussions presented at this conference on leprosy have been most interesting to a mycologist, but I fear the mycologist has few technics to offer the leprologist in his long continued attempt to cultivate *M. leprae* in the laboratory.

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Dr. Emmons. I would like to call now on Dr. Louis Diamond, who is here because of certain interesting historical occurrences. In 1909, as Dr. Binford reminded me this morning, Dr. Clegg reported the cultivation of what he believed was Hansen's bacillus in mixed culture with amebae and bacteria. A year later Dr. Duval pointed out that this bacillus would grow in the medium in the presence of bacteria whether or not the amebae were present. Now you may not accept this as a culture of the leprosy bacillus, for it may have been only one of the many acid-fasts that have been cultured since. In any case, because of Dr. Diamond's experience and success in producing a culture medium that supports the growth of amebae, we would like to have his comments in this program at this time. Dr. Diamond is in the Parasitology Laboratory of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

Resume of Dr. Diamond's Discussion

Dr. Diamond stated that he hesitated to discuss at a conference on leprosy research the development of a technic for the axenic cultivation of another recalcitrant organism, *Entamoeba histolytica*, because, aside from the fact that Clegg in 1909 (*Philippine J. Sci.* **4** (1909) 77) claimed to have cultivated the leprosy bacillus in association with *Entamoeba histolytica*, it and *Mycobacterium leprae* appear to have little in common. A statement, however, that had been made by Dr. Hanks in answering a question by Dr. Goldman had induced him to discus the subject (see page 476).

In 1959 when Dr. Diamond was confronted with the problem of growing *E. histolytica* in the absence of any other living organisms, there were 36 years of failure behind him. Many protozoologists had tried their hand and had failed. Obviously, he pointed out, not all the combinations had been tried. Specifically the problem that was faced in 1959 was this: *E. histolytica*, the single cell protozoan which causes amebic colitis and amebic hepatitis in man, had never been cultivated continuously *in vitro* in the absence of metabolizing bacteria, fungi, protozoa, or metazoan cells. Several technics had been developed for short term cultivation, but no technic had been devised that would permit indefinite subcultivation. The objective was to iso-

late amebae, and get them to grow under axenic conditions in such a way that they could be maintained indefinitely in subculture.

Four sources of amebae were available. First, there was fecal material that could be obtained from infected patients hospitalized in the Clinical Center at the National Institutes of Health. Second there were cultures of amebae in which the bacterial flora consisted of several unidentified species. Third, there were monoxenic cultures in which amebae were grown with a single species of bacteria. Fourth, there were monoxenic cultures in which the associate of the amebae consisted of another protozoan, in this case either one or two members of the family *Trypanosomatidae*. One of these organisms was *Trypanosoma cruzi*, a pathogen of man. The other was a species of *Crithidia* isolated from the gut of a hemipteran. The ameba-crithidium cultures were chosen for several reasons. Foremost of these was the fact that the crithidium was the easiest of the associates to eliminate from the system.

Dr. Diamond pointed out that, despite the use of cold logic in making the choices, fate and luck played important roles. For example in 1958, Maj. Donald L. Price gave Dr. Diamond a *Hemiptera* that carried a flagellate in its feces. Dr. Diamond succeeded in cultivating this flagellate, which was a new species of *Crithidia*, not realizing at the time that it was to play a most vital part in devising a method for axenic cultivation of the *E. histolytica*.

Later, Dr. Diamond found that this new species of *Crithidia* was an excellent associate for supporting the growth of *E. histolytica*. In retrospect it appears that this organism provides the amebae with some nutrient that enables them to go from monoxenic to axenic growth.

In developing a medium for growing *E. histolytica* axenically, Dr. Diamond decided to make use of ingredients readily available in any well-equipped tissue culture or microbiologic laboratory rather than seek out exotic ingredients. In part, his decision was influenced by the work of Weinstein and Jones (*J. Parasitol.* **42** (1956) 215-236). These investigators used ingredients of the same order to axenically cultivate a parasitic nematode. Dr. Diamond also took the approach that the microbial associates commonly used to support *in vitro* growth of *E. histolytica* could possibly be serving as vehicles bringing nutrients in proper form to the amebae and not as suppliers of specific enzyme systems (Porter. *Ann. Rev. Microbiol.* **7** (1953) 273-294).

TABLE 1. *Ingredients of nutrient broth (TTY) used in preparation of diphasic medium for axenic cultivation of* Entamoeba histolytica *(pH adjusted to 7.2 with 1 N NaOH; autoclaved 10 min. 15 lbs., 121°C).*

Ingredient	Amount
Tryptose (Difco)	1.00 gm.
Trypticase (BBL)	1.00 gm.
Yeast extract (BBL)	1.00 gm.
Glucose	0.50 gm.
L-cysteine hydrochloride	0.10 gm.
Ascorbic acid	0.20 gm.
NaCl	0.50 gm.
KH_2PO_4	0.08 gm.
K_2HPO_4 (anhydrous)	0.08 gm.
Water (distilled)	80.00 ml.
Resazurin (0.05% aq. sol.)	0.20 ml.

TABLE 2. *Composition of diphasic medium for axenic cultivation of* Entamoeba histolytica.

Agar slant:	
TTY[a]	80.0 ml.
Horse serum (inactivated)	20.0 ml.
Agar	1.6 gm.
5 ml. per tube (screw-capped, 16 × 125 mm.)	
Overlay:	
TTY (diluted with distilled water 1:4) or	
Distilled water, each containing 0.05% agar	75.0 ml.
$CEEM_{25}$, $CEEH_{25}$ or CEE_{25}[b]	20.0 ml.
Vitamin mixture NCTC-107[c]	5.0 ml.

[a]TTY = Tryptose, trypticase, yeast extract nutrient broth.
[b]$CEEM_{25}$ = Chick embryo extract medium 25%.
$CEEH_{25}$ = Chick embryo extract heated 25%.
CEE_{25} = Chick embryo extract 25%.
[c]NCTC = National cancer tissue culture medium.

Using lantern slides Dr. Diamond showed the audience the composition of the medi-

um he had used to cultivate *E. histolytica* axenically (Diamond, *Science* **134** (1961) 336-337).

Dr. Diamond emphasized that luck again played an important role in the work. It happened that the first amebic strain used for the experiment was much easier to cultivate independently than another strain tried later. He suggested that those trying to cultivate *M. leprae* might be equally fortunate in picking a strain that was easy to grow. Dr. Diamond said that after his group had succeeded in growing the amebae in a medium containing chick embryo they removed this ingredient and still obtained growth. *E. histolytica* can now be isolated and maintained in a clear, liquid medium free of chick embryo extract.

Dr. Diamond closed his discussion by remarking that in spite of the statement by Dr. Hanks that all possible ways to grow *M. leprae in vitro* had been tried it might be that the right combination of ingredients has not been put together in a medium or the right procedure has not yet been used.

Dr. Emmons. Thank you, Dr. Diamond. May we return now to Dr. Ulrich's paper. Are there questions, or comments on Dr. Ulrich's presentation?

Dr. Hanks. Dr. Ulrich, your findings on the amino acid patterns during systemic fungal infection may be pertinent to the problems of genetic susceptibility. Since these atypical patterns were found during infections, I wonder if it was possible to ascertain if they persisted after recovery. In other words, was this a genetic property?

Dr. Ulrich. No. Because of the way in which we get our specimens, and the fact that many of the patients come from outside areas, these were run just at the time the isolations were made and we have had no follow-up yet. In some of these patients, however, we also ran blood levels of the free amino acids and did find some unusual patterns. However, we find that it is much better to study the urine patterns. Because of the clearance rate, urine magnifies small differences that are difficult to find in serum. Thus far we have not followed patients whose disease has been cleaned up by the use of antifungal agents. This is yet to be done. It may be that even though we do clean them up it may not change their physiology.

Dr. Hanks. I did not want to comment further while we were discussing the interesting work Dr. Ulrich has presented, but I would like later, when you are ready, to comment on the work of Dr. Diamond.

Dr. Emmons. You may do so now.

Dr. Hanks. Dr. Diamond, no one has asked me what I think about the cultivation problem. The question asked was what have people done in the past. That is all I undertook to answer. Let me assure you that I would have quit this business long ago if I did not think it interesting, fascinating, challenging and possible. Now, the sort of thing you showed is a wonderful example of the well-conceived approach plus serendipity. The cogitation I would offer concerning the interesting mixture of components in your medium is not strictly in respect to your problem but for all of us. When we use peptones, gelatins, hydrolysates and derivatives that are made commercially in large amounts we may sometimes expect magic that cannot happen if we go to the host species and derive the same kind of components aseptically. The reason is that these commercial products usually have been inhabited by microbes during the course of preparation. For example, Eilizabeth Work has shown that bacteriologic peptones contain diaminopimelic acid. Such compounds are unrelated to the host from which the peptone material was derived. During simplification and analysis of your medium, you quite possibly might find that there have been contributions by unsuspected microbes. If so, it would be exciting to know what these have been.

Dr. Diamond. I am well aware of this, Dr. Hanks. The problem that faced us was to induce the amebae to grow free from other living cells, so that we could do these things. I could have started, as some have

done in this type of work, with very simple compounds and worked with salts and balance the salts and then go to the adverse types of amino acids and so forth. But it occurred to me that this might take a very long time. So I approached it the other way, with the idea that once we had got them growing we could start to define the media and learn new facts. This is actually what we are doing in the laboratory.

Dr. Reich. Dr. Diamond, do your cultures retain pathogenicity?

Dr. Diamond. That is an interesting question. We do not know. We are testing them right now. That is all I can tell you. I suspect that they do not retain pathogenicity. But I also think that it is possible to restore it by certain manipulations that we are trying right now. That is as much as I can say.

Dr. Reich. I would like to point out an anomaly that we labor under, going back to the criteria of classification. We pointed out that acid-fastness is the absolute one. There are two other minor or controversial possibilities. These would be nerve invasion and lepromin reactivity, but how these will respond with pure culture is a major question. So we are faced with the problem of isolating an organism in order to prove the isolation characteristics.

Dr. Binford. Dr. Diamond, we were greatly pleased that you finally decided that you would come and talk about cultivating a large organism with a group that was attempting to cultivate a small one. You have put a ray of hope into the program, which I expect will bear fruit. My predecessor, Dr. Doull, used to say that one of these days somebody was going to cultivate the leprosy bacillus not realizing that it could not be cultivated. We hope that out of this meeting today new approaches will come. This straight forward report of your mental processes in this achievement has been most stimulating.

Dr. Emmons. We will have to close the session at this point.