SCHEMA FOR THE DIFFERENTIATION OF *NOCARDIA ASTEROIDES* AND *NOCARDIA BRASILIENSIS*

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The tests usually employed for the identification of the *Nocardia* species do not give good results in many cases. The colonial morphology as a taxonomic criterion is too variable and is influenced by the type of nutrients in the culture medium used. Furthermore, the sensitivity to chemotherapeutic compounds is not a reliable diagnostic criterion, since variation is great between strains (Mackinnon *et al.*, 1958). Recent studies on physiological activity, with casein and tyrosine decomposition and gelatin hydrolysis, appear to give more promising results in this respect (González-Ochoa, 1945; Gordon and Mihm, 1957; Schneidau and Shaffer, 1957; Mariat, 1958; Bojalil *et al.*, 1959).

In preliminary studies it has been observed that important differences exist between the growth of *Nocardia asteroides* and *Nocardia brasiliensis* in a dilute gelatin medium that contains this protein as the sole source of nitrogen and carbon.

This paper summarizes observations made on the type of growth, substrate utilization, intermediate substances formed (amino acids), and pH changes in the medium by different species of *Nocardia*.

MATERIALS AND METHODS

Strains. The organisms used were received from various institutions and are listed in table 1. All the strains were grown on Sabouraud's culture medium and reclassified in this laboratory on the basis of tyrosine and casein decomposition, gelatin hydrolysis, and acid production from different sugars (Bojalil *et al.*, 1959).

Culture medium. The culture medium used to establish the type of growth was prepared as follows: gelatin, 4.0 g; distilled water, 1000 ml; pH 7.0; after the adjustment of pH it was sterilized by autoclaving and poured into tubes measuring 16 by 150 mm, in quantities of 5 ml. Each series of tubes was inoculated with a colony fragment of approximately 1 mm in diameter from the growth obtained on Sabouraud's agar, and incubated at 28 C for 5, 10, 15, 20, and 25 days; growth (quantity and type), pH and presence of amino acids was recorded.

Marked differences were observed after 20 days of incubation, and the data described herein correspond to this time. In some series the inocula were washed with 0.85 per cent saline solution, but it was not considered necessary to continue this procedure since the results were similar to those obtained in the series which were cultured directly.

In quadruplicate series the following tests were verified. (1) pH determination:—In order to observe pH variations, 1 or 2 drops of a 0.04 per cent bromothymol blue solution was added to each tube. In the majority of cases a determination of pH values was also made with a potentiometer (Beckman G. S. model).

(2) Presence of amino acids:—In another series the presence of amino acids was determined using a 0.25 per cent ninhydrin (1,2,3-triketohydrindene; Eastman) solution in butanol saturated with phosphate buffer pH 7.4, 0.15 M. One ml of this solution was added to the seeded tubes, heated to 60 C in a water bath for 30 min, during which time the tube was shaken frequently, and then left to stratify in the oven for 6 hr at 37 C. The appearance of a blue-violet ring on the surface of the medium indicated the presence of α-amino groups. This was not observed in the uninoculated gelatin medium that was used as control. Readings were made at 5, 10, 15, and 20 days of incubation at 28 C.

In another series of tubes inoculated with 1.0 mg wet weight of Nocardia, the consumption of substrate was determined by the quantitative biuret method (Colowick and Kaplan, 1957). Hopkins tubes were used in the determination of mycelial mass. Readings were made at 2, 5, 7, 11, 15, and 20 days after inoculation in samples of 1.0 ml of cell-free culture medium to which 4.0 ml of biuret reagent had been added allowing

TABLE 1

*List of Nocardia species used**

Laboratory No.	Name when Received	Source†
20	*N. asteroides*	ISET
50	*N. asteroides*	ISET
3399	*N. asteroides*	ISET
1160	*N. asteroides*	ISET
151	*N. asteroides*	ISET
652	*N. asteroides*	ISET
1109	*N. asteroides*	ISET
383	*N. asteroides*	ISET
9956A	*N. asteroides*	ISET
19	*N. asteroides*	ISET
1602	*N. asteroides*	ISET
694	*N. asteroides*	IHM
789	*N. asteroides*	IHM
1406	*N. asteroides*	IHM
1457	*N. asteroides*	IHM
583	*N. asteroides*	IMRU
427	*N. asteroides*	IMRU
W3661	*N. asteroides*	IMRU
529	*N. asteroides*	IMRU
420-1	*N. asteroides*	IMRU
420-2	*N. asteroides*	IMRU
1669	*N. asteroides*	CDC
549	*N. asteroides*	CDC
347	*N. asteroides*	CDC
129	*N. asteroides*	MC
130	*N. asteroides*	MC
131	*N. asteroides*	MC
132	*N. asteroides*	MC
R-217	*N. asteroides*	UPHG
C-98	*N. asteroides*	UPHG
1913	*N. brasiliensis*	ISET
479	*N. brasiliensis*	ISET
477	*N. brasiliensis*	ISET
416	*N. brasiliensis*	ISET
1005	*N. brasiliensis*	ISET
22	*N. brasiliensis*	UPHG
23	*N. brasiliensis*	UPHG
24	*N. brasiliensis*	UPHG
36	*N. brasiliensis*	UPHG
37	*N. brasiliensis*	UPHG
38	*N. brasiliensis*	UPHG
39	*N. brasiliensis*	UPHG
40	*N. brasiliensis*	UPHG
41	*N. brasiliensis*	UPHG
54	*N. brasiliensis*	UPHG
55	*N. brasiliensis*	UPHG
56	*N. brasiliensis*	UPHG
57	*N. brasiliensis*	UPHG
852	*N. brasiliensis*	UPHG
766	*N. brasiliensis*	UPHG
180	*N. brasiliensis*	UPHG
52	*N. brasiliensis*	UPHG
35	*N. pasteuroides*	ISET
98	*N. convoluta*	ISET
4063	*N. rhodnii*	IHM
2392	*N. transvalensis*	IMRU
547	*N. rhodnii*	FMUSP
1139	*N. phenotolerans*	UC
3409	*N. polychromogenes*	ATCC
8674	*N. minima*	ATCC
4273	*N. corallina*	ATCC
616	*N. corneus*	ISET
4064	*N. leishmanii*	IHM
145	*N. melanosporus*	IHM
2391	*N. pretoriana*	IMRU
2356	*N. globerula*	ATCC
9911	*N. gypsoides*	NHI (ISET)
6846	*N. blackwellii*	ATCC

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† Meaning of abreviations: ISET = Instituto de Salubridad y Enfermedades Tropicales, México, D. F.; IHM = Instituto de Higiene de Montevideo, Uruguay; IMRU = Institute of Microbiology, Rutgers University, U. S. A.; CDC = Communicable Disease Center, Atlanta, Ga., U. S. A.; MC = Mayo Clinic, Rochester, Minn., U. S. A.; UPHG = Unidad de Patología, Hospital General, México, D. F.; IOCB = Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; ISPB = Instituto de Saude Publica de Bahía, Brazil; FMUSP = Facultade de Medicina, Da Universidade de São Paulo, Brazil; UC = Universidad de Colombia, Bogotá, Col.; ATCC = American Type Culture Collection, U. S. A.

it to sit for 30 min at room temperature and recording the optical density at 540 mμ. The fall in optical density indicated the consumption of substrate as compared with a gelatin control curve. Only 10 strains were studied by this method, 5 of *N. asteroides* and 5 of *N. brasiliensis*.

In addition, another series of tubes was seeded

with variable quantities of inoculum (1.0, 10^{-1}, 10^{-3}, and 10^{-5} mg wet weight) to determine if variations in type of growth, pH, or presence of amino acids depended on the quantity of inoculum.

RESULTS

The type of growth, as illustrated in figure 1, is different for the two species studied. *N. asteroides* grows very poorly in the gelatin medium. It develops a thin flaky type of growth that is easily dispersed throughout the medium, and on rare occasions forms a slight surface film that later settles to the bottom.

N. brasiliensis develops to a much greater extent, forming rounded colonies which form a pellicle, adhere strongly to the wall, or sink to the bottom in a mass. The formation of aerial mycelium in the surface colonies is evident.

In the series inoculated with 1.0, 10^{-1}, 10^{-3}, and 10^{-5} mg wet weight of mycelium, a similar pattern of growth was observed to that which had been described previously. All the strains of *N. brasiliensis* were capable of development even in the tubes inoculated with the least concentration (10^{-5}); in some cases it was possible to observe slight growth in those tubes inoculated with the greater concentration of some strains of *N. asteroides*.

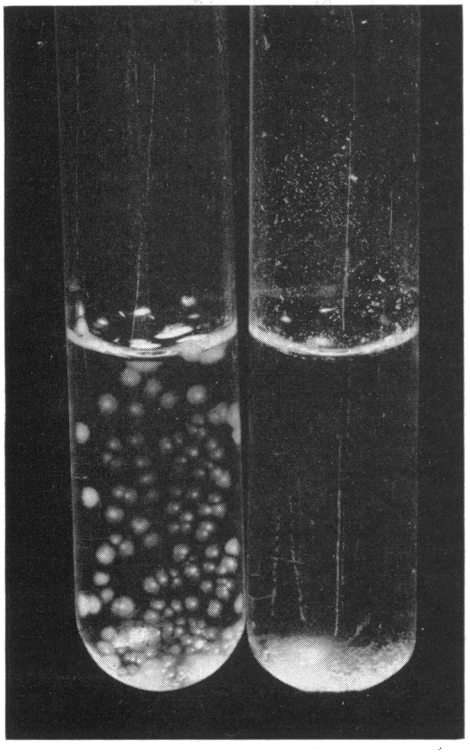

Figure 1. Variation in growth of *Nocardia brasiliensis* and *Nocardia asteroides* in a dilute gelatin medium. *Left tube: N. brasiliensis* develops spherical colonies. *Right tube: N. asteroides* develops scanty growth that is flaky.

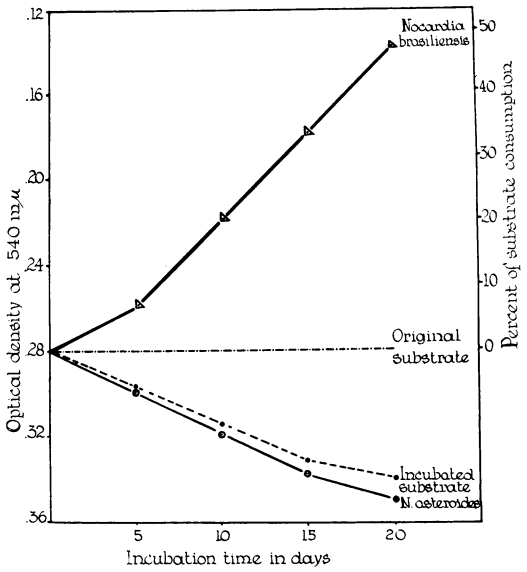

Figure 2. Gelatin consumption by *Nocardia brasiliensis* and *Nocardia asteroides*. Determined in cell-free culture medium by the fall in optical density with biuret reagent.

The determination of pH shows that all the strains of *N. brasiliensis* alkalinize the culture medium during growth, turning the indicator blue, whereas the majority of the *N. asteroides* strains acidify the medium, turning the indicator yellow. In some cases the pH remained unchanged with the *N. asteroides* cultures.

The potentiometric readings revealed a gradual rise in pH with *N. brasiliensis* from 7.0 to 8.0; and a fall from 7.0 to 5.8 with *N. asteroides*.

The presence of amino acids, as detected with ninhydrin, was positive for all strains of *N. brasiliensis* from 15 to 20 days. This test was negative for all strains of *N. asteroides*; only two of them gave a slight violet tint at 20 days. This reaction was minimal in comparison with that obtained with *N. brasiliensis*.

The data given in figure 2 reveal that the consumption of substrate, as determined by the fall

TABLE 2

Characteristics of Nocardia asteroides and Nocardia brasiliensis in dilute gelatin medium

Species	Growth			Colony Development	pH Changes			Ninhydrin Reaction	
	Negative	Scarce	Abundant		None	Acid	Alkaline	Positive	Negative
N. asteroides (30 strains)	18	12	0	0	10	20	0	0	30*
N. brasiliensis (22 strains)	0	1	21	22	0	0	22	22	0

* Two strains gave a slight violet tint.

TABLE 3

Characteristics of several Nocardia species in dilute gelatin medium

Strains and Laboratory No.	Growth	Colony Formation	pH Variation	Ninhydrin Reaction	Final Classification
N. pasteuroides 35	Negative	−	Ac	−	*N. asteroides*
N. leishmanii 4064	Negative	−	Ac	−	*N. asteroides*
N. pretoriana 2391	Scarce	−	V	+	Not classified
N. rhodnii 4063	Negative	−	N	−	*N. asteroides*
N. rhodnii 547	Scarce	−	N	−	*N. asteroides*
N. gypsoides 9911	Negative	−	Ac	−	*N. asteroides*
N. phenotolerans 1139	Negative	−	N	−	*N. asteroides*
N. transvalensis 2392	Scarce	−	N	−	*N. asteroides*
N. convoluta 98	Negative	−	N	−	*N. asteroides*
N. corneus 616	Negative	−	N	−	*N. asteroides*
N. melanosporus 145	Negative	−	N	−	*N. asteroides*
N. polychromogenes 3409	Dubious	−	N	−	*N. asteroides*
N. globerula 2356	Negative	−	Ac	−	*N. asteroides*
N. blackwellii 6846	Negative	−	Ac	−	*N. asteroides*
N. corallina 4273	Scarce	−	Ac	+	Not classified
N. minima 8674	Scarce	−	N	−	*N. asteroides*

− = Negative; + = positive; N = neutral; Ac = acid; V = variable.

in optical density with biuret reagent, gradually rose with *N. brasiliensis*. It reached 47 per cent after 20 days of incubation. It was not possible to detect substrate consumption by this method with the *N. asteroides* strains. A slight increase in optical density was observed in some cases for this species in comparison with the control. The results of these experiments are summarized in table 2.

The characteristics of *N. leishmanii*, *N. rhodnii*, *N. blackwellii*, *N. gypsoides*, *N. transvalensis*, *N. convoluta*, *N. polychromogenes*, *N. globerula*, *N. minima*, *N. pasteuroides*, *N. phenotolerans*, *N. corneus*, and *N. melanosporus* were similar to those obtained with *N. asteroides*. On the other hand, *N. pretoriana* did not grow well but produced amino groups. The pH was sometimes changed to the alkaline side (blue). *N. corallina*, did not grow well. It did not produce alkaline changes in the pH, but gave a positive ninhydrin reaction (table 3).

DISCUSSION

Gelatin hydrolysis, as demonstrated by methods that utilize precipitating agents, has given satisfactory results in the hands of several investigators (McDade and Weaver, 1959). Nevertheless, there are differences of opinion in reference to the results obtained by these methods when studying *N. asteroides* (Gordon and Mihm, 1957; Schneidau and Shaffer, 1957; Mariat, 1958; Bojalil *et al.*, 1959). The use of a gelatin-containing culture medium as the only source of N and C, the detection of intermediate breakdown

products, substrate consumption, and final pH, demonstrate that it is feasible to separate the Nocardia pathogens into two groups (see Differential Schema).

DIFFERENTIAL SCHEMA FOR THE IDENTIFICATION OF *NOCARDIA ASTEROIDES* AND *NOCARDIA BRASILIENSIS*

Nocardia
(Acid-fast, fragmenting microorganisms)
Cultures in Gelatin Medium
(0.4 per cent, pH 7.0)

Growth: Develops spherical colonies. Colonies on the surface wall, and bottom	Growth: Development of scanty flakes or no development
pH: Alkaline	pH: Acid or neutral
Ninhydrin reaction: Positive	Ninhydrin reaction: Negative
Utilizes substrate	Does not use substrate
Nocardia brasiliensis	*Nocardia asteroides*

One group of microorganisms is capable of utilizing gelatin as the only source of N and C, breaking it down into amino acids, and alkalinizing the medium. All the strains classified as *N. brasiliensis* belong to this group.

The other group of organisms is not capable of utilizing gelatin under the conditions of the experiment. All strains classified as *N. asteroides* belong to this group.

Other Nocardia such as *N. leishmanii*, *N. gypsoides*, *N. transvalensis*, *N. convoluta*, *N. polychromogenes*, *N. globerula*, *N. blackwellii*, *N. minima*, *N. pasteuroides*, *N. phenotolerans*, *N. corneus*, *N. melanosporus*, and *N. rhodnii* have a growth pattern similar to that of *N. asteroides*. They did not produce amino groups, and failed to change the pH indicator to the alkaline side. These data are in accord with previous reports on physiological activity (González-Ochoa and Sandoval, 1956; Schneidau and Shaffer, 1957; Bojalil *et al.*, 1959).

On the other hand, *N. pretoriana* behaves in a manner similar to *N. brasiliensis* with respect to amino acids production and pH change of the medium; but it does not develop in the same form as does *N. brasiliensis* and the pH reaction is not consistent. However, this species has been considered to be *N. brasiliensis* by other authors (González-Ochoa and Sandoval, 1956; Gordon and Mihm, 1959). *N. corallina* presents characteristics of both groups, but the fact that it does not grow well and does not change the indicator to an alkaline pH, makes us think that it does not belong to the *N. brasiliensis* group since these two characteristics are very constant for that group.

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SUMMARY

The type of growth, substrate utilization, amino acid formation, and pH changes in a dilute gelatin medium (0.4 per cent in distilled water) has been investigated. Thirty strains of *Nocardia asteroides*, 22 strains of *Nocardia brasiliensis*, and 16 strains of *Nocardia* species were studied.

The results obtained suggest that the Nocardia can be divided into two different metabolic groups. One group develops round colonies which strongly adhere to the wall and bottom of tubes, utilizes gelatin as the only source of N and C, breaks down gelatin into amino acids, and produces an alkaline medium. All the strains classified as *N. brasiliensis* belong to this group.

The other group, under the same experimental conditions grows very poorly in the gelatin medium and develops a thin flaky type of growth that is easily dispersed throughout the medium. These reactions are characteristic of the strains classified as *N. asteroides* and most of the other *Nocardia* species studied.

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