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IDENTITY OF THE FUNGAL SYMBIONT OF *SIREX NOCTILIO*

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[*Manuscript received December* 30, 1968]

Summary

The fungus associated with *S. noctilio* F. in Australia is identified as *Amylostereum areolatum* (Fr.) Boidin. By using a polystyrene film to prevent the breakup of the chains of arthrospores during staining, it was possible to demonstrate that the spores are homokaryotic though usually multinucleate. Viable homokaryons were established from arthrospores by using a tissue culture medium incorporating growing callus tissue of *Pinus radiata*. Homokaryons and heterokaryons of the *S. noctilio* fungus were opposed in all possible combinations against homokaryons and heterokaryons of *A. chailletii* (Pers. ex Fr.) Boidin, *A. laevigatum* (Fr.) Boidin, and *A. areolatum*, the only known species of *Amylostereum*. The *S. noctilio* fungus was interfertile only with *A. areolatum*, as judged by production of anastomoses, clamped mycelium, and fertile fruit bodies. Starch-gel electrophoresis showed that the soluble proteins of the *S. noctilio* fungus corresponded more closely with those of *A. areolatum* than with those of *A. chailletii*.

I. Introduction

The fungus associated with *Sirex noctilio* F. in Australia was identified as a species of *Amylostereum* Boidin by Talbot (1964), who studied fructifications which were produced in culture. Stillwell (1960, 1962, 1966) reported that the fungus associated with *S. juvencus* L. and *Urocerus* spp. in Canada had been identified by Dr. M. K. Nobles, and is *Stereum chailletii* Fr. [*Amylostereum chailletii* (Pers. ex Fr.) Boidin]. King (1966) compared the Australian *Sirex* fungus with *A. chailletii*, introducing protein patterns obtained by starch-gel electrophoresis as a point of comparison in addition to the morphological characters used previously, and suggested that the *S. noctilio* fungus might be a strain of *A. chailletii*, although differences were apparent both in the cultural characteristics and in the protein patterns of the two fungi. Neither of the other two species of *Amylostereum* [*A. areolatum* (Fr.) Boidin and *A. laevigatum* (Fr.) Boidin] was included in the comparison.

Amylostereum species are heterothallic (Boidin 1958). Both Talbot (1964) and King (1966) stressed the need for interfertility tests between the *S. noctilio* fungus and the known species of *Amylostereum*, and pointed out that such tests could not be performed until monosporous cultures could be obtained. However, it was difficult to grow the *S. noctilio* fungus beyond spore germination, so that further work on homokaryotization was regarded as a prerequisite for further taxonomic studies.

Boidin (personal communication) finds it a problem to distinguish *A. chailletii* and *A. areolatum* by their fruit bodies, but states that these species are intersterile and may be recognized in culture by the presence of arthrospores in *A. areolatum*

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and their absence in *A. chailletii*. Evidently the morphology of the fruit body did not identify the species to which the *S. noctilio* fungus belongs. The presence of arthrospores in culture (Talbot 1964) suggests that the *S. noctilio* fungus is *A. areolatum*. It is unwise, however, to rely on a single character for specific determination, particularly as the fungus associated with siricids in Canada has already been determined as *A. chailletii*. A more detailed study, including interfertility tests, was necessary before ascribing either name to the *S. noctilio* fungus in Australia.

II. Cultures and Sources

Homokaryotic cultures of the three known species of *Amylostereum* were obtained from Professor J. Boidin, University of Lyon, France (Table 1). Heterokaryons of *A. chailletii* had been obtained previously from Dr. M. K. Nobles, and one (Waite Institute No. 154) was used in these studies: it was derived from a fruiting body from Algonquin Park, Ontario, Canada. Dikaryons of both *A. areolatum* and *A. laevigatum* were obtained by crossing homokaryons of compatible mating type. Only one dikaryotic isolate (P2) of the *S. noctilio* fungus was used in most of this investigation, because it is the one from which the only viable homokaryons (PH1, PH2, PH3, and PH4) were obtained. Isolate P2 was obtained from wood around a recent *Sirex* oviposition tunnel in a pine tree on the Pittwater Plantation near Hobart, Tas.

Table 1
Sources and mating types of *Amylostereum* spp. from France

Species	Lyon Accession Number	Homokaryon Type	Source
A. areolatum	LY 4922	A1B1 A2B2 A1B2 A2B1	Fruit body on a conifer, le Boreon, Alpes-Maritimes, France
A. chailletii	LY 4893	1 2	Fruit body on *Picea abies*, Alpes-Maritimes, France
	LY 4622	1 2	On *Abies alba*, Bizanos, Basses-Pyrenees, France
A. laevigatum	LY 4905	A1B1 A2B2 A1B2 A2B1	Fruit body on *Juniperus communis*, col de Braus, Peira-Cava, Alpes-Maritimes, France

III. Homokaryotization of the S. noctilio Fungus

Three methods are available for separating the component monokaryons of a dikaryotic basidiomycete: single-spore isolation, microsurgery, and the use of toxic chemicals. The first two methods have been reviewed by Miles and Raper (1956): single-spore isolation is applicable only in species whose spores contain only one nuclear type; microsurgery (Harder 1927) can be used only for species where clamp formation is the normal method of septation, and where hyphae are sufficiently large for the operation to be practicable. Toxic chemicals used for this work can be divided into two classes on the basis of their mode of action. Miles and Raper (1956) used cholic acid and sodium taurocholate, which have a specific effect on the dikaryon in disrupt-

ing normal clamp formation; the hook cell fails to fuse with the peg on the penultimate cell, but both continue growing, and since both hook cell and penultimate cell are homokaryotic at this stage, two homokaryotic branches of different polarity are formed. It is still necessary to separate these homokaryons from one another, either by maceration, or by isolation of hyphal tips. The toxic chemicals ($CuSO_4$, $ZnSO_4$, etc.) used by Kerruish and DaCosta (1963) are perhaps preferable, since they seem to select for only one nucleus of the original pair, and thus massive isolations can be made from the edge of a growing colony without recourse to blending or micromanipulation.

Although the basidiospores of the *S. noctilio* fungus are uninucleate (Talbot 1964) and would be suitable for production of homokaryons, King (1966) was unable to obtain growth beyond the germling stage despite persistent attempts using a wide range of media. The cells of this fungus are too small for the microsurgical technique to be applicable.

Arthrospores are much more readily available than basidiospores, and it was suggested (Talbot, personal communication) that attempts should be made to obtain homokaryons from these spores. Thus it was necessary to investigate the nuclear content and mode of formation of the arthrospores, to see if they could be expected to give rise to homokaryons.

The arthrospores arise by fragmentation of the aerial mycelium into long chains of 60 or more spores; clamp connections usually remain clearly visible. Each dikaryotic cell can divide into about 10 arthrospores, each containing 1–6 nuclei. If the first stage of arthrospore formation is the laying down of a septum between the original pair of nuclei, then the arthrospores must necessarily be homokaryotic; but if the original nuclei divide before septation occurs, the daughter nuclei might intermingle and the arthrospores could then be heterokaryotic. To decide which of these alternatives occurs it was necessary to maintain the arthrospores in their chains during the rigorous Giemsa–HCl procedure for staining nuclei. A method was developed using polystyrene film; this is based on the method of Lingappa and Lockwood (1963) for collecting fungal propagules from soils. A 2% solution of polystyrene was prepared by dissolving 1 g of plastic Petri-dish chips in 50 ml benzene–toluene (2 : 1). One drop of this solution was smeared evenly over one side of a microscope slide, making sure that it overlapped the edges (this greatly helped the film to stick to the glass throughout the subsequent staining). When the film was almost dry (3–5 sec, detectable by the changing interference patterns) the slide was lowered on to the aerial mycelium of the culture to be investigated, so that the mycelium stuck in the drying film. The slide was then removed and set aside for at least 5 min to allow the film to dry thoroughly. The slide, film, and adhering mycelium were then taken through the Giemsa–HCl staining procedure. As usual, the technique had to be tailored to suit the fungus concerned; in this case the only critical details were time in and temperature of the hot HCl. Five min ($\pm$5 sec) at 60°C ($\pm$2 degC) are the optimum conditions, variations from which render the staining inadequate.

Arthrospores were found to contain 1–6 nuclei, and some dikaryotic cells were seen in which a cross wall was being laid down between the two nuclei before any nuclear division had occurred. However, this was observed only rarely, and could not be considered as conclusive evidence that all arthrospores would be homokaryotic.

Further evidence was obtained by marking the limits of a growing colony on an agar plate at 2-day intervals, so that it was possible to isolate arthrospores from areas of colony whose age was accurately known. These spores were stained, and the nuclear content of 500 spores from each age group was recorded; the percentage of spores containing any particular number of nuclei was calculated, and a graph was plotted to show how this percentage varies with age (Fig. 1). This shows that most spores, if not all, are initially uninucleate, and therefore must be homokaryotic.

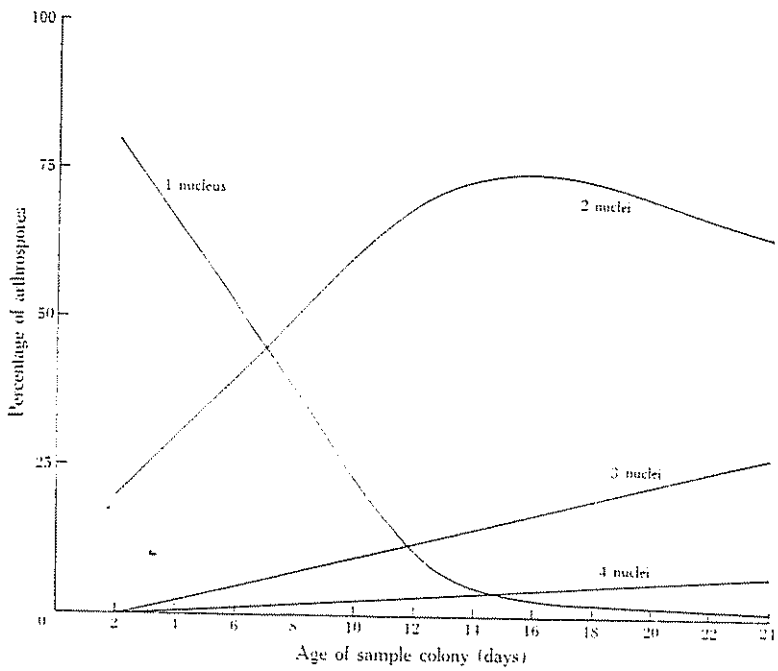

Fig. 1.—Variation of nuclear number between arthrospores of different ages.

Initial attempts to obtain viable homokaryons from arthrospores were as fruitless as previous attempts using basidiospores. The spores germinated readily, and gave rise to a mycelium devoid of clamp connections, but growth ceased at an early stage, before the colony became visible to the naked eye. Use of a wide range of both natural and artificial media, including supplementation with vitamins, failed to improve growth. Incorporation of activated charcoal in the media, to remove growth inhibitors which might have been present, improved growth for a short time, but still failed to yield any viable homokaryons. Arthrospores placed on Cellophane overlying growing dikaryotic cultures failed to germinate. Finally, a tissue culture medium* containing dispersed callus cells of *Pinus radiata* was tried, and by its use four viable homokaryons were eventually obtained. The factors involved in this successful germination have not yet been elucidated; but once the homokaryons had become established they

* This medium contained the minerals of Murashige and Skoog (1962) combined with the organic constituents of Harvey (1967) but with twice the normal kinetin concentration.

grew quite satisfactorily, though slowly, on 2% malt agar.

None of the toxic chemicals used by Kerruish and DaCosta (1963) was effective with this fungus. Many other chemicals were tried, including fungicides, phenols, and antibiotics; although some caused the fungus to produce abnormal hyphae, sometimes with pseudoclamps, sometimes with simple septa, growth always reverted to the dikaryotic type when transfers were made to non-toxic media.

For two reasons sodium taurocholate was not used to produce homokaryons for this study. Firstly, a preliminary experiment indicated that clamp formation in the *S. noctilio* fungus was not disrupted to the extent described by Miles and Raper (1956) for some other fungi. Secondly, the major problem seems to lie in maintaining homokaryons rather than obtaining them, and from this point of view the method offers no advantages over arthrospore isolation which is technically simpler.

IV. Anastomosis

The taxonomic value of anastomosis is uncertain, and must remain so until the nature and extent of genetic control over the phenomenon is established. Bourchier (1957) showed that environmental conditions such as temperature, and composition and pH of the culture medium, may all affect the frequency of hyphal fusion. Thus, as a species criterion, hyphal anastomosis is of value only in providing supplementary information, and that only in cases where anastomosis does occur. Because of the difficulty of obtaining homokaryons of the *S. noctilio* fungus, and because of the similarity between *A. chailletii* and *A. areolatum*, it was decided to introduce anastomosis as a supplementry criterion in this investigation.

Small inocula about 1 mm diameter were cut from the growing edges of colonies on 2% malt agar, and were opposed about 2·5 mm apart in pairs on the undersides of sterile coverslips which were placed on sterile cavity slides so that the inocula were suspended in the cavity. The coverslips were sealed to the slides with sterile distilled water which was introduced by capillary attraction, thus forming a humid chamber. The slides were stacked in a large covered dish, kept moist, and incubated at 16–23°C. Because of the short duration of each investigation, no problem was encountered with contamination. Each slide was examined under the microscope daily, or more frequently if anastomosis seemed imminent. This method of observing anastomoses is thought to have several advantages over those where the fungus grows in a nutrient medium: in the present method the hyphae from opposing inocula are almost forced into contact with one another because they grow predominantly in one plane over the surface of the cover slip; the cells at points of contact are not directly in contact with any growth medium, and so possible adverse effects of medium or pH may be lessened; the fact that most hyphal contacts occur on the underside of the coverslip makes observation and photography of anastomosis relatively easy, and also simplifies the task of tracing hyphae back to the parent inocula to confirm identity.

Homokaryons and heterokaryons of *A. chailletii*, *A. areolatum*, *A. laevigatum*, and the *S. noctilio* fungus were paired in all possible combinations; each pairing was repeated at least three times. Results were very clear: anastomosis was observed at least once in every possible combination of the *S. noctilio* fungus with *A. areolatum;* anastomosis was not seen in any of the other pairings.

V. Dikaryotization

It is generally agreed that the formation of a regularly clamped mycelium is the minimum evidence required for the unequivocal establishment of conspecificity in those basidiomycetes which possess clamps (Mounce and Macrae 1936, 1937, 1938; Nobles 1943; McKay 1959; Boidin and des Pomeys 1961). Whether this clamped mycelium is formed from two compatible homokaryons, or from a homokaryon and a heterokaryon is not important, for it has been shown that the dikaryotization of a homokaryon by a heterokaryon—the Buller Phenomenon (Buller 1931)—is as good evidence of conspecificity as the formation of a heterokaryon from two homokaryons (Terra 1959; Boidin and des Pomeys 1961; Nobles and Frew 1962; Raper 1966). The essential feature of either process is that two compatible nuclei, capable of conjugate division, are brought together in one cell, and manifest their presence by producing hyphae bearing clamped septa. Sometimes the mycelium formed may bear only sparse, often incomplete, clamps; but as it is uncertain what significance should be given to this phenomenon, the essential criterion for conspecificity is the formation of a stable, regularly clamped mycelium. If this mycelium is capable of producing fertile fruit bodies, this is further corroborative evidence.

In this series of experiments, homokaryons of *A. chailletii*, *A. areolatum*, *A. laevigatum*, and the *S. noctilio* fungus were paired on 2% malt agar in all possible combinations; isolations were made from the area where the two colonies met in each case. Homokaryons were also paired with heterokaryons in all combinations; in these cases isolations were made from points on the homokaryon colony furthest away from the area of contact of the two colonies, taking the necessary precautions against isolating from intrusive growth of the original dikaryon. Each pairing was repeated at least three times; cultures were examined daily, and isolations made from them at irregular intervals:

Table 2

Results of crosses between the *S. noctilio* fungus and *A. areolatum*

+ = True clamp formation. × = Sparse, often pseudoclamp formation. − = No clamp formation

A. areolatum	*S. noctilio* fungus				
	P2	PH1	PH2	PH3	PH4
4922 dikaryon		×	×	×	×
4922 A1B1	+	−	−	−	−
4922 A2B2	+	+	+	+	+
4922 A1B2	+	×	×	×	×
4922 A2B1	×	×	×	×	×

No clamps or pseudoclamps were seen in any of the pairings between members of the three known species, confirming this as a valid criterion for separating the species; neither were they seen in pairings of the *S. noctilio* fungus with *A. chailletii* nor with *A. laevigatum*. However, either clamps or pseudoclamps were found in almost every cross between the *S. noctilio* fungus and *A. areolatum*. The results of the last-mentioned crosses are summarized in Table 2, and are explicable if one assumes an

incompatibility factor genotype of A1B1+A2B3 for the *S. noctilio* fungus isolate P2, and if one assumes that all four homokaryons derived from it have the A1B1 nucleus. The *A. areolatum* dikaryon was formed by mating 4922 A1B2 with 4922 A2B1, which explains the incompatibility of this dikaryon with any of the homokaryons. The *S. noctilio* fungus homokaryons are wholly compatible with only the A2B2 strain of *A. areolatum*, while only the A2B1 strain of *A. areolatum* contains factors which are incompatible with both nuclei of the *S. noctilio* fungus dikaryon.

VI. Interfertility

Although tests for production of fertile fruit bodies are not generally included in so-called "interfertility tests", it is clear that the greatest affinity between two nuclei is indicated by their ability to undergo karyogamy and then meiosis, as is inherent in the production of basidia and basidiospores. One cannot accept Macrae's (1967) description of conjugate division as "consummation" of the relationship between two individuals; it is simply evidence of dikaryotization. The true climax of a close relationship is interfertility, which is characterized not by the production of clamp connections, but by the formation of fertile fruiting structures. Interfertility is the ultimate criterion of conspecificity over and above the criterion of dikaryotization, although the latter is always considered adequate.

Both Talbot (1964) and Boidin (personal communication) have indicated that a morphological comparison of the fruit bodies of *A. chailletii*, *A. areolatum*, and the *S. noctilio* fungus would not yield sufficient information to enable the latter fungus to be placed in either of these two species, firstly because of differences which occur with age, and secondly because of the apparent similarity between *A. chailletii* and *A. areolatum*. Thus the present study was initiated not with the intention of examining the fruit bodies in detail, but to discover which isolates fruited most readily, and to find out if the *S. noctilio* fungus $\times$ *A. areolatum* crosses are fertile.

The wood-block method for producing fruit bodies (Tamblyn and DaCosta 1958) has been used successfully in this laboratory for many years, and was the method used predominantly in this work. In this case only *Pinus radiata* sawdust was used in the enriched sawdust medium. The sawdust medium was also used in deep crystallizing dishes, initially with the intention of casing with soil after a few weeks incubation, but this was later found to be unnecessary. Wood-block cultures and dish cultures were inoculated with the *S. noctilio* fungus, *A. areolatum*, *A. chailletii*, and all those crosses of *A. areolatum* with the *S. noctilio* fungus in which clamps or pseudoclamps had been observed. Homokaryons were also used to check the possibility that they could form fruit bodies by themselves.

The results of this investigation were strikingly clear-cut. All cultures which fruited did so both in the dishes and on the wood-blocks; the former method was much more rapid than the latter (fructifications appearing within 8 weeks, as compared with 16–20 weeks for the wood-block method) and was entirely satisfactory for this experiment. Abundant fruiting structures were formed by the *A. areolatum* dikaryon and by all (*S. noctilio* fungus $\times$ 4922 A2B2) dikaryons; these fruit bodies were essentially as described by Talbot (1964), being composed of skeletal and clamped generative hyphae, cystidia, and basidia bearing four basidiospores each. An attempt was made to obtain single-basidiospore cultures from a fruit body of the dikaryon (PH4 $\times$ 4922

A2B2) but the germination problem was again encountered; only one basidiospore grew into a viable macroscopic colony, and a back-cross proved this to be of the parental A2B2 mating type. Fructifications were also produced by two of the dikaryons bearing pseudoclamps, namely (PH3 × A1B2) and (PH2 × A2B1); however, while these fructifications appeared normal to the naked eye, they were found to be microscopically abnormal: both contained skeletal and generative hyphae and abundant cystidia, but the hyphae bore simple septa and pseudoclamps as well as apparently normal clamps, and in neither case were any basidia found. None of the homokaryons fruited.

VII. Electrophoresis

As techniques have improved, protein separation and resolution have also improved, and the electrophoretic patterns of enzymes and other proteins are rapidly becoming useful as criteria in taxonomy, supplementary to the morphological criteria on which fungal taxonomy is largely based. The value of protein patterns in fungal taxonomy has been recently discussed (Clare, Flentje, and Atkinson 1968), and a novel method of expressing differences between the patterns of different isolates has been presented (Whitney, Vaughan, and Heale 1968).

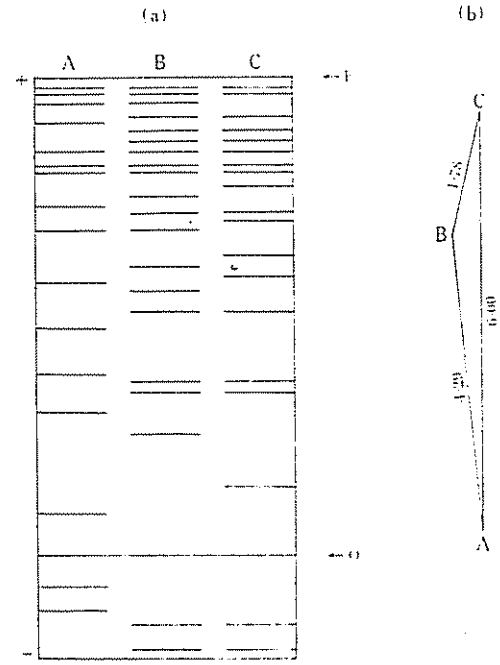

Fig. 2.—(*a*) Protein patterns in starch gel after electrophoresis at pH 9·5. *A*, *Amylostereum chailletii*; *B*, *S. noctilio* fungus; *C*, *A. areolatum*. *F*, Anodic front. *O*, Origin. (*b*) Relationship between the three fungi as expressed by the method of Whitney, Vaughan, and Heale (1968).

It was considered desirable to use this technique in this study because King (1966) suggested that the *S. noctilio* fungus might be a strain of *A. chailletii* on the basis of similarities between the protein patterns of the two fungi. Culture, extraction, electrophoresis, and staining for proteins were performed as described by Clare, Flentje, and Atkinson (1968) with the following exceptions: cultures were grown in a 2% solution of Difco malt extract, and were harvested after 6 days; optimum pH for all buffers was found to be 9·5.

Diagrammatic scale drawings of the protein patterns of *A. areolatum*, *A. chailletii*, and the *S. noctilio* fungus are shown in Figure 2(*a*). A total of 20 bands was visible in the *S. noctilio* fungus extract, 19 in *A. areolatum*, and 16 in *A. chailletii*. The protein pattern of the *S. noctilio* fungus is not identical with that of the particular isolate of *A. areolatum* used, but 14 of the bands correspond; on the other hand, the *S. noctilio* fungus shows only 7 bands in common with *A. chailletii*. Figure 2(*b*) shows these results as interpreted by the method of Whitney, Vaughan, and Heale (1968), and indicates that the *S. noctilio* fungus is much closer to *A. areolatum* than to *A. chailletii*. As all the other tests described in this paper have shown that the *S. noctilio* fungus is *A. areolatum*, the slight differences which are apparent between the electrophoretic protein patterns should be regarded as expressions of the normal range of intraspecific variation within *A. areolatum*.

VIII. Acknowledgments

I would express sincere thanks to Drs. P. H. B. Talbot and J. H. Warcup, who supervised this work, and to Dr. B. G. Clare for his interest and advice with the electrophoresis. I would also like to thank Professor J. Boidin for his continued interest and for supplying cultures, and the National Sirex Fund Committee for financial assistance.

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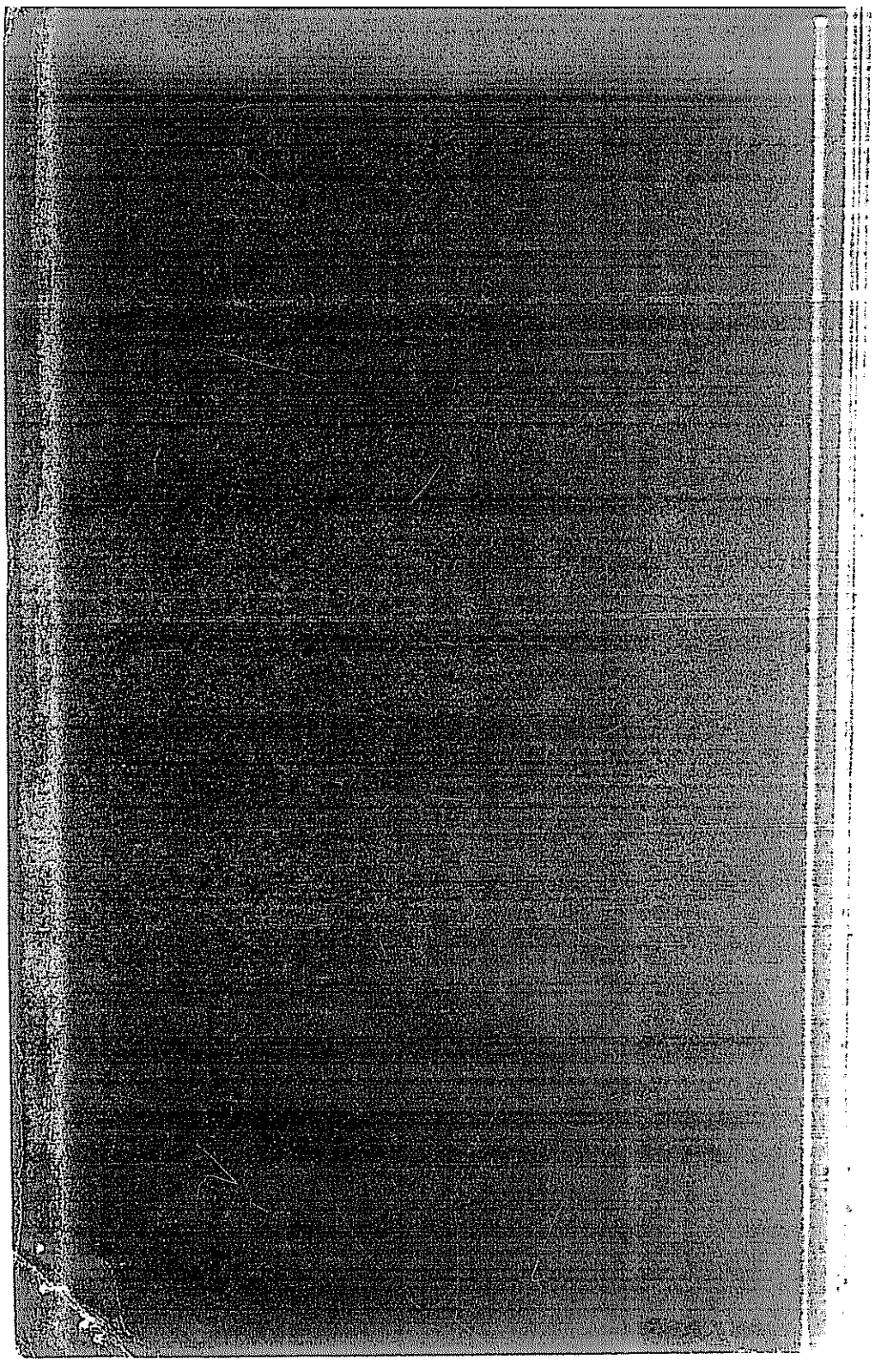

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