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Application of crossed light and humidity gradients for the investigation of slime-molds

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In last few years the gradient methods were commonly used for the quick estimation of life requirements of microorganisms (bacteria and algae). Gradients of different concentrations of antibiotics (Szybalski 1952)) as well as gradients of pH (Sachs 1956) and different concentrations of Fe and Cu ions (Weinberg 1957) were applied to the estimation of the effectiveness of antibiotics. Interesting results were also obtained by Halldal (1957, 1958) and Halldal and French (1958) who applied crossed gradients of light and temperature in algae research. The gradient method consists in sowing out microorganisms upon the whole gradient area, with the subsequent determination of the zone of their optimal development.

The unsufficient knowledge as to the life requirements of slime-molds, stimulated the author for applying to their study the method of the so-called crossed gradients. The ability for migration upon the surface of media creates in slime-mold research much larger possibilities for the application of the above method, than for instance, in studies of immobile microorganisms, — as the moving plasmodia can actively choose optimal zones for their development.

Among various factors which determine the development of slime-molds, light and humidity are two of those most important. The present paper describes the method of the crossed gradients of light and humidity and its application to the study of such characteristics of slime-molds as the ability for migration, regeneration of sclerotia, fructification etc.

METHODS

Reactions of slime-molds were studied on agar media without any nutrient added. The media were placed into cells consisting of a glass plate at the bottom, and a box with a glass upper wall, serving as a cover of the cell (Fig. 1). To the bottom plate (*a*) a glass frame (*b*) is fastened by means of araldite, reducing the dimensions of the cell to 100 × 100 mm. This frame is filled up with an agar layer (*c*). The upper part of the cell is built out of plexiglass frames — 5 mm. thick, which are painted black on the outer side (*g*). The chamber is covered with

a glass pane (*e*) which in turn is tightly fitted to the plexi frames. On this pane a light filter (*h*) is placed. After the cell is closed its edges are tightened with vaseline (*f*).

Light gradient — was obtained by placing the cells inside a light thermostat (Rakoczy 1962). A neutral filter (*h*) with a gradually increasing blackening on the surface, was placed upon the upper

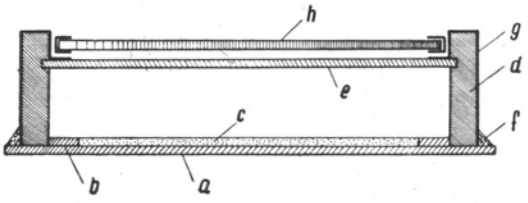

Fig. 1. Schematic cross-section of the cell

a — basic glass plate; *b* — glass frames; *c* — agar layer; *d* — plexiglass walls; *e* — upper glass pane; *f* — vaseline sealing; *g* — black varnish; *h* — filter

wall of the cell. This created such conditions inside the illuminated cell in which one side of the agar plate received the maximal light intensity, whereas the opposite side was almost completely blacked out.

The light filters were prepared out of Foton photographic plates — 14/10 DIN, suitably exposed. The edges of plates were covered with black paper strips, which together with the black painting of the cell walls allowed the surface of the agar media to be illuminated only with light which passed through the filter. Using filters with various grades of blackening, different steepness of the light gradient can be obtained.

The measurement of the light gradient was performed once for each kind of filter. This is done in the light thermostat and consists in moving the upper part of the cell with the filter, upon a 1 × 3 cm. slit,

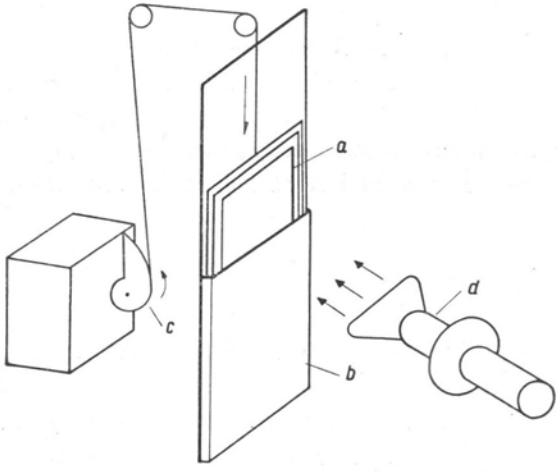

Fig. 2. Arrangement for drying agar plates

a — agar plate; *b* — box; *c* — the cam for the regulation of speed of the plate movement; *d* — hair dryer

under which a photocell is placed, connected to the galvanometer. Each filter can be thus calibrated in terms of the percentage of the initial light intensity which reaches particular spots on the agar plate.

The humidity gradient — is obtained by means of appropriate drying of the agar plate, prepared previously by pouring out 10 ml. of the 1,3 per cent of the agar solution onto the above described glass plate, which must be slightly warmed up and placed precisely in a horizontal position. After the agar layer is solidified the plate is placed in a vertical position in a device, presented on Fig. 2, which shifts it gradually into the box (*b*). The part emerging upon the edge of the box is continuously dried in a stream of warm air from a hair-dryer. The speed of shifting the plate into the box is regulated by means of a clock mechanism and a cam (*c*). This results in a comparatively quick movement of the plate at the beginning of the drying process,

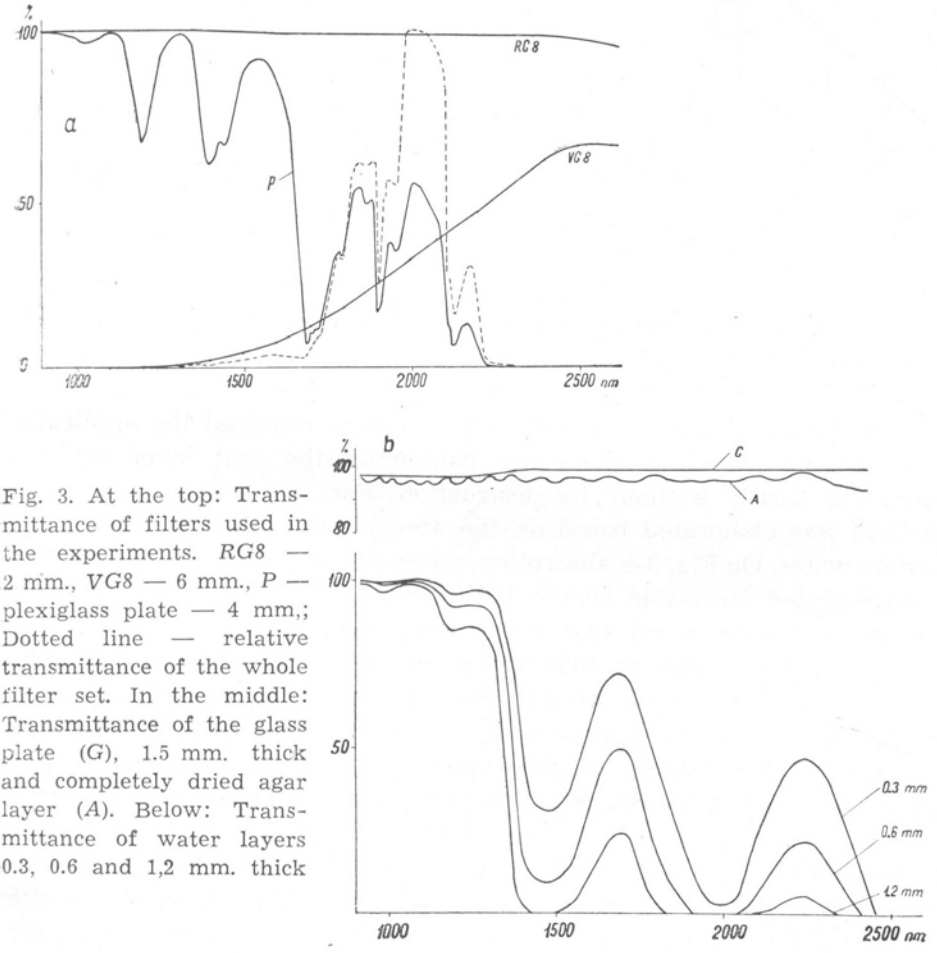

Fig. 3. At the top: Transmittance of filters used in the experiments. *RG8* — 2 mm., *VG8* — 6 mm., *P* — plexiglass plate — 4 mm.; Dotted line — relative transmittance of the whole filter set. In the middle: Transmittance of the glass plate (*G*), 1.5 mm. thick and completely dried agar layer (*A*). Below: Transmittance of water layers 0.3, 0.6 and 1,2 mm. thick

which later on slows down. As the drying affects only this part of the agar plate which is emerging out of the box, the described above procedure allows to obtain a gradually increasing degree of dessication on the

plate — from the almost undessicated lower part to the almost completely dried up upper part. The upper part of the agar plate is exposed to the warm air stream for the longest time, and hence its high degree of dessication. In case of a too weak dessication the treatment can be repeated several times. The speed of the movement of the plate, the shape of the cam, as well as the distance of the hair dryer and the tem-

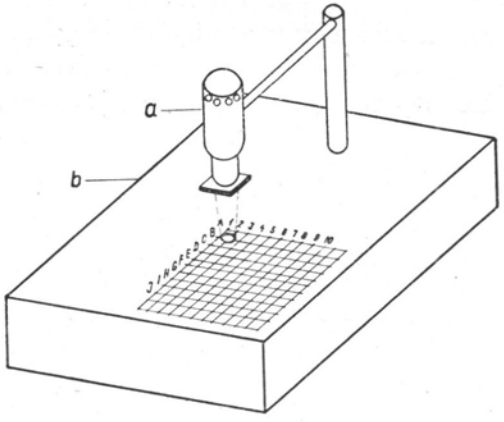

Fig. 4. Arrangement for the measurement of local absorption of infra-red radiation by the plates: *a* — cover of the microscopic lamp; *b* — the table with aperture, with a thermocouple below and a net of drawn lines which serve for setting up the plate in a proper position

perature of the air stream must be determined experimentally. In order to obtain a uniform effect in the drying procedure the hair dryer must be placed exactly in axial position and not to close to the agar surface.

The measurement of the humidity gradient required the application of a method by which the water content of the agar layer could be estimated locally without its destruction. For this purpose an optical method was elaborated based on the absorption of the infra red radiation by water. On Fig. 3-*b* absorption curves at the range of 1000—2500 mμ are presented for water layers: 0,3, 0,6 and 1,2 mm. thick. The water content of the prepared agar plates remained in about the same range of values. Absorption at 1400—2400 mμ. wave length, changes considerably in water layers of different thickness, whereas in the same conditions, absorption in glass and agar is quite unimportant (Fig. 3-*b*).

For the measurment of the water content, the agar plates were irradiated with infra-red at 1700—2200 mμ. wave length, generated by a common electric bulb, equipped with filters RG8 — 2 mm., VG8 — 6 mm., and a plate of plexiglass 4 mm. thick. The relative transmission curves of particular filters, as well as of a whole filter set, are presented on Fig. 3-*a*. Measurements of absorption were carried out by means of an arrangement, presented in Fig. 4. A microscopic lamp equipped with a 6V./30Watt bulb, was used as a radiation source. After passing the filters, the light was concentrated at a spot of ca 10 mm. in diameter — at the level of the table *b*. It reached

the detector (a thermocouple Kipp and Zonen), connected to the galvanometer. The agar plate was slowly shifted across the table, which was previously covered by a tiny net of parallel lines, drawn at a distance of 1 cm. from each other. This facilitated considerably the measurements of transmission at different spots on the plate.

For determinations of the absolute water content, the measuring instrument must be previously calibrated. This was done by the measurement of transmission of the agar plates, which were uniformly

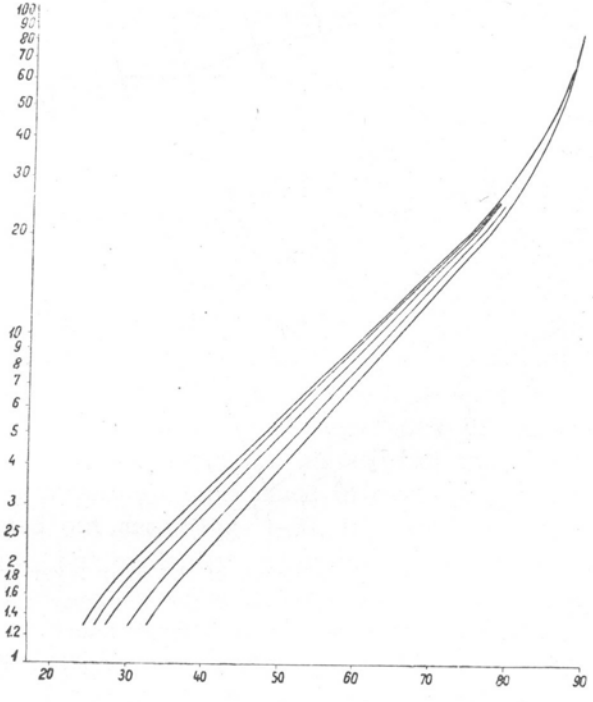

Fig. 5. The calibration curve

X-axis — percentage of infra-red transmittance, *Y*-axis — percentage of agar concentration

dried up. Their water content was at the same time estimated by weight. Results of calibration were put together in tables in order to facilitate computations. They are presented in graphs on Fig. 5. There are several curves, according to the initial transmission of the agar plates, which amounted to 24—26 per cent. An example of the distribution of the humidity gradient on the agar plate after drying, is represented on Fig. 6*a*. These differences arise from the fact, that even before drying the thickness of the agar layer was not uniformly distributed over the whole area of the plate.

The procedure for preparation of the plates was as follows:

a. Pouring out and subsequent cooling of the agar.
b. Measurements of local transmissions of infrared light.

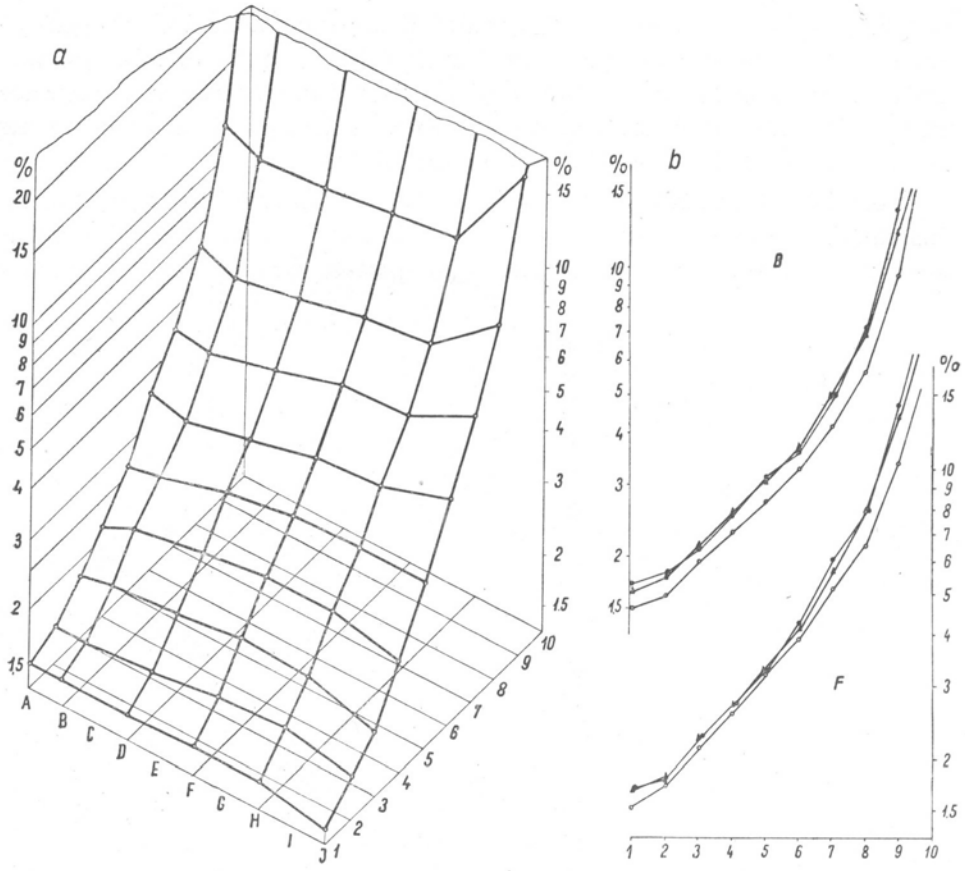

Fig. 6. *a*: Humidity distribution of the agar layer (vertical axis — % of agar plate after drying. *b*: The stability of the humidity gradient measured along lines *B* and *F*, immediately after drying, after 48 hours, and after 4 days. X — axis points of measurements, Y axis — humidity in % of agar

c. Drying.

d. Measurements of local transmission values at the same spots.

e. Computation of the percentage of agar concentration at the examined spots.

Stabilization of the gradient is quite sufficient if we avoid minute differences in temperature, which might exist within the cell. The initial difficulties, caused by the condensation of small drops of water on the upper glass pane inside the cell were overcome by the installation of a small ventilator inside the thermostate, which blowed air just upon the plate. The humidity gradient did not considerably change during the first 48 hours following the preparation of the plates. Fig. 6*b* represents the distribution of humidity gradients just after dessication of the agar layer, as well as after 48 hours and 4 days.

MATERIAL

Preliminary experiments for studies of the response of slime-molds on the different light and humidity conditions were carried out by the gradient method on plasmodia and sclerotia of *Physarum nudum* Macbr. The appropriate culture conditions, required by this species, were already described in an earlier paper (R a k o c z y 1962).

Examples for the application of gradients

1. Migration of the plasmodium in the light gradient

Considering the results, obtained in a previous work (R a k o c z y 1962), concerning the correlation between the fructification and the age of the slime-mold culture, a very young — 4 days old — plasmodium was used for the illustration of the influence of various light intensities on its behaviour. A comparatively large piece of the plasmodium was placed in the middle of the agar plate, containing only pure medium, without nutrient. The cells with plasmodia were then exposed to 1220 lux light intensity in a light thermostat (R a k o c z y 1962). After a certain time, regeneration of the plasmodia take place, and they spread almost uniformely in all directions over the agar surface (Fig. 7-*A*). In about 6 hours time, and sometimes even earlier, the process of regeneration is finished and the plasmodia begin to move over the media. The migration of a young plasmodium took place in over 90 per cent of cases, towards the blacked out area (Fig. 7-*B*). It usually migrates upon the surface of the plate, but evidently avoids spots wich are subjected to more intense illumination. After 3—4 days, when the plasmodium is still strong enough, and does not die from starving, it also migrates sometimes to the illuminated area, where it forms fruiting bodies, normally on the glass edge of the plate, and only very rarely on the surface of the agar.

2. The regeneration of sclerotia in the humidity gradient

Sclerotia of *Physarum nudum* were obtained by a simple method of dessication of plasmodia, described by J u m p (1954). Small fragments of sclerotia were placed on the agar plates, at spots with different degree of humidity, and the regeneration process was followed by a photographic method. At spots with a considerable amount of moisture, complete regeneration takes place in 2—3 hours, whereas on agar, dried up to 5 per cent, the time of regeneration is much more prolonged, and an accumulation of the protoplasm in the middle of sclerotia is there visible for a long time. If the concentration of agar is

increased by drying up to 9—12 per cent, the regeneration proceeds longer than 12 hours, and it stops completely if the agar is still more dessicated. When using a very steep humidity gradient plasmodia, derived out of sclerotia situated at the border of a very dry agar area, regenerate excentrically, expanding mainly towards regions of greater humidity, and clearly avoiding more arid zones on the media.

3. Fructification of the slime-mold upon the crossed light and humidity gradients

As was already stated in an earlier work — the slime-mold — *Physarum nudum* — requires light for fructification. The often observed formation of fruiting bodies on the glass, provides evidence, that also the humidity of the substrate plays here an important role. The application of the crossed gradients of light and humidity of the substratum, created for the slime mold studied, an ample intensity range of both factors, acting together. Accordingly to the previously obtained evidence, 12 days old plasmodia were used in the experiments, in order to obtain a quicker effect of fructification.

A large piece of plasmodium was placed within the moist area of the agar plate. After regeneration it starts migrating to the more illuminated zone (Fig. 9-*A*). It behaves in this respect in quite an opposite manner as a young plasmodium. In the illuminated zone the plasmodium remains for about 24 hours, forming fruiting bodies at strongly dried up spots (Fig. 9-*C*). In case, when the dessicated area of the agar plate is comparatively large (eg. about a half of the surface), the plasmodium usually avoids places most strongly dried. Nevertheless it happens sometimes, that when a very great plasmodium is used, it migrates also into arid zones on the medium. In such cases it usually forms sclerotia, as the return to more humid areas is then impossible, because of the loss of water. On strongly dessicated plates it was also observed that plasmodia which migrated too far into the arid zone, have formed sclerotia in parts, directed towards the most dry spots of the agar media, whereas the rest of the plasmodium returned back to the more humid zone.

DISCUSSION

The life requirements of slime-molds in the vegetative (plasmodium) state, as well as factors, governing the process of fructification, are not yet sufficiently well known. Our information in this respect is based on laboratory experiments and on the observations in nature. Among a great variety of factors which influence the process of fructification (eg. pH of the medium, character of the substratum, temperature etc.), very essential are light and humidity of the medium. The role of light

in the process of fructification was already investigated by many authors (Gray 1938, 1953, Lieth 1954, 1956, Straub 1956 etc.). There is nevertheless little known about the influence of humidity. Apart from scattered informations, which can be found in various papers dealing generally with the fructification of slime-molds, only Schure (1949) has worked out a special method of their culture at different conditions of moisture content in the media. The cultures are carried out in pots which are placed into larger beakers, with a small water layer at the bottom. Accordingly to the height of the pot, the humidity of the air, etc., a certain humidity gradient is established upon the walls of the pot, which are gradually more and more moistened towards the bottom. Schure's method was also used by Sobels (1950) in her studies on the fructification of slime-molds with coloured plasmodia. This method however does not allow to determine exactly the moisture content in the medium, though it gives quite satisfactory results in investigations of the fructification process. It has therefore only a qualitative character.

The method described by the author has this advantage, that it allows an exact evaluation of the moisture content in the medium. Schure's method also differentiates to some extent the light conditions (as on inner walls of the pot illumination is always weaker), but such differences are very difficult for a quantitative estimation. We often find in nature the plasmodia of slime-molds in full developmental state, in humid and dark evironment (eg. under the bark of decaying trunks, over the soil in the woods etc.). On the contrary, sporangia, or plasmodia in prefructification stage, appear rather in dry and illuminated places. It looks therefore quite clear, that in the first stage of development, plasmodia exhibit a negative phototaxy and a positive hydrotaxy, whereas in the period preceeding fructification, they show quite opposite reactions.

As in natural environment stronger illumination and greater dessication are usually interdependent, it is difficult to judge on the basis of sole observations in nature, which of those two factors is more important for the development of slime-molds. Although Howard (1931) and Seifriz and Russel (1935) deny the role of light in the process of fructification, arguing, that rather darkness than light enhances it, it is already found beyond doubt, that for coloured plasmodia (eg. *Physarum polycephalum*) light represents a condition *sine qua non* for passing from the vegetative into the generative phase. The negative phototaxy, observed at the young plasmodia in the light gradient, comply satisfactorily with the observations in nature — confirming at the same time results obtained in experiments on the influence of the age of plasmodia on fructification (Rakoczy 1962).

The application of light gradient with the use of chromatic light,

creates new possibilities for the elaboration of an active spectrum of phototaxy of plasmodia at different stages of their development. Accordingly to many authors (Cayley 1929; Gilbert 1928), the humidity of the substratum exerts considerable influence on the morphology of sporangia. Moreover Gilbert (1928) claims that the percentage of germinating spores is much greater if they were formed in conditions which prevented plasmodia from loosing water too quickly. However at present we are not able to say exactly in what direction, to what extent and at what conditions of the moisture content, these changes take place. Maybe the gradient method will provide a proper tool for solving these and other problems concerning the development of plasmodia, as well as other life requirements of slime-molds in general.

SUMMARY

The present work contains the description of a method for obtaining crossed gradients of light and humidity of the substratum, for studies of slime-molds. The light gradient was obtained by using filters, with gradually decreasing density which were prepared out of photographic plates. The filters were placed upon a flat cell used for experimentation. The humidity gradient was obtained by drying the agar layer, poured off on a glass plate — 10×10 cm. The drying process was performed in a special device, which shifted gradually the plate under a stream of warm air. Local measurements of the moisture content in the media were carried out by estimating the absorption of infra-red radiation.

The paper presents examples of the different reactions of slime-molds (such as migration, regeneration of sclerotia, and fructification) — at different conditions of light and humidity, as studied by the gradient method.

The author is greatly indebted to Professors Dr F. Górski and Dr J. Zurzycki for their valuable advice and help during this work as well as to Dr Kontik from the Physics Department of the Institute of General Chemistry in Warsaw, for the measurements of infra-red absorption spectra in glass, plexiglass and agar.

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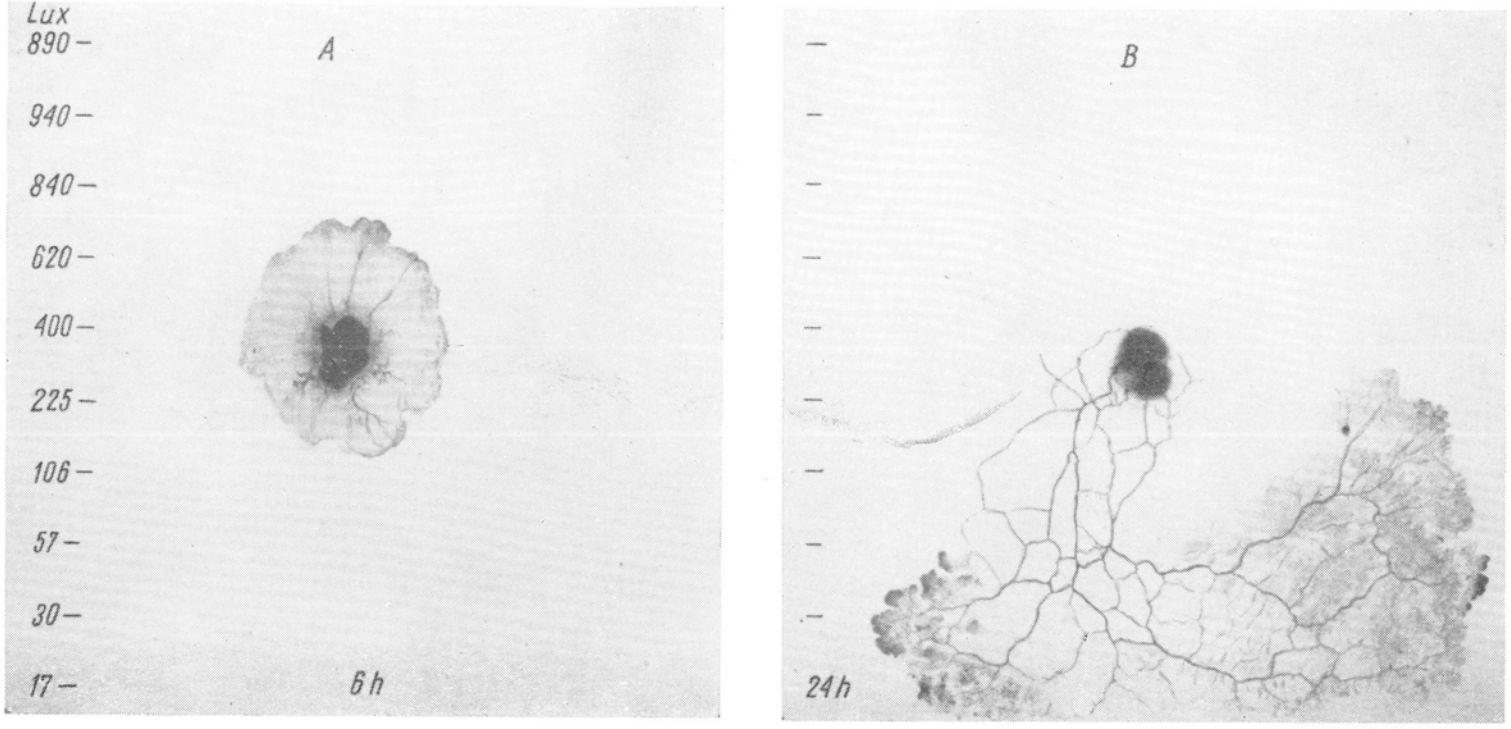
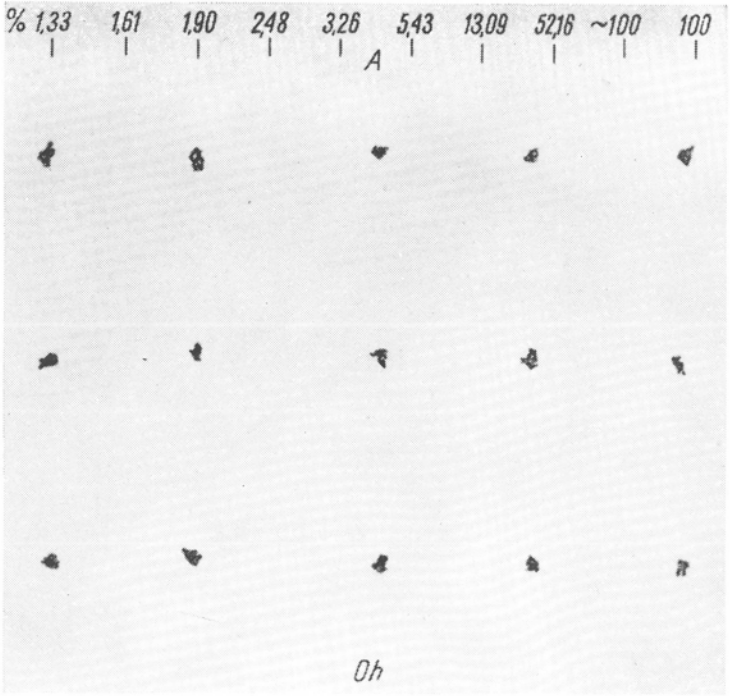


Fig. 7. Migration of plasmodia within the light gradient:
A — after 6 hours; *B* — after 24 hours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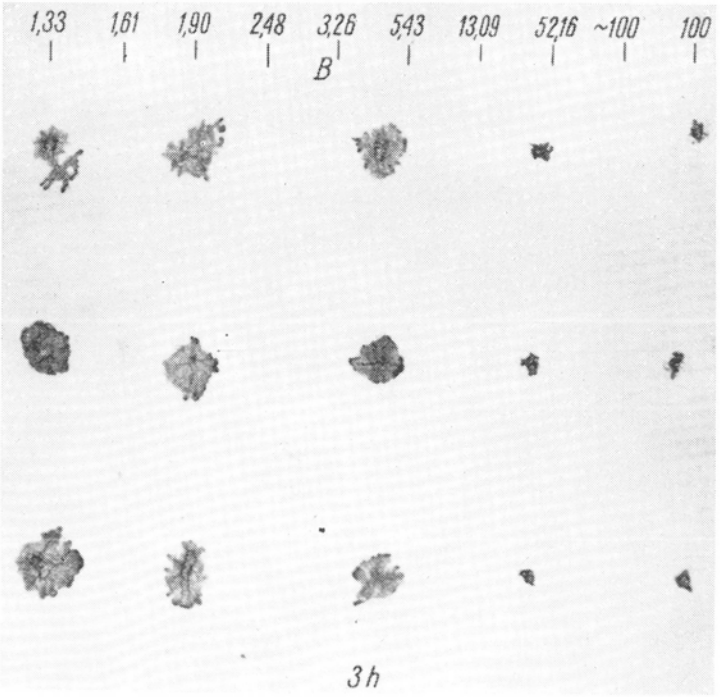
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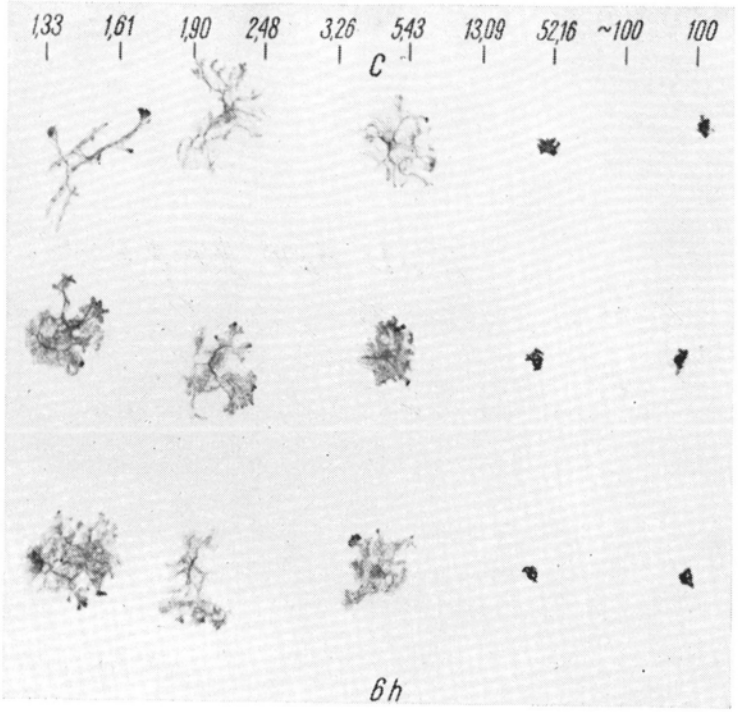
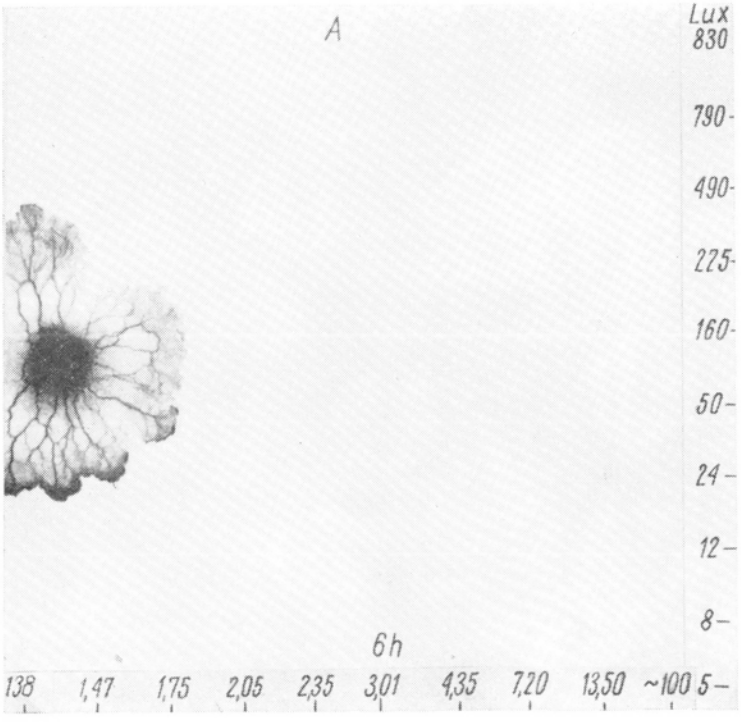


Fig. 8. Regeneration of sclerotia within the humidity gradient

A — immediately after placing sclerotia on the agar plate; *B* — after 3 hours; *C* — after 6 hours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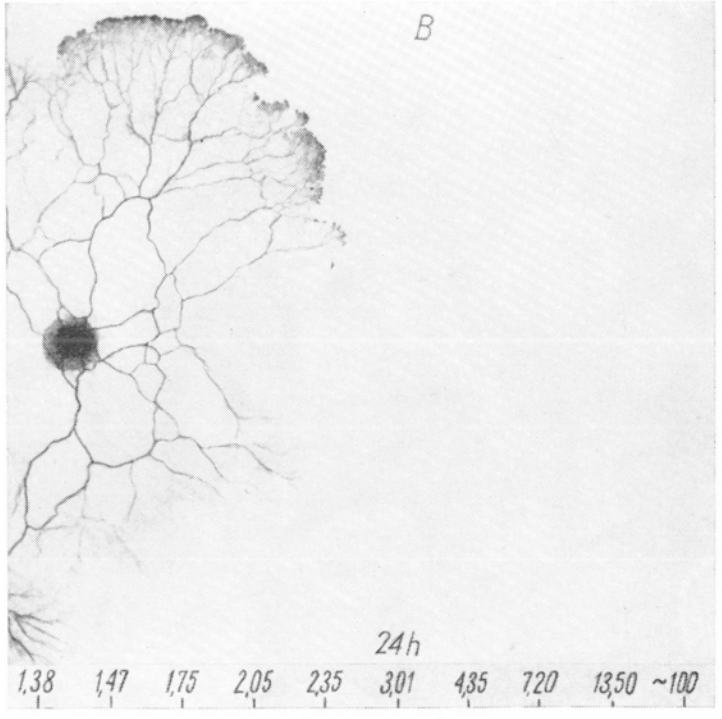
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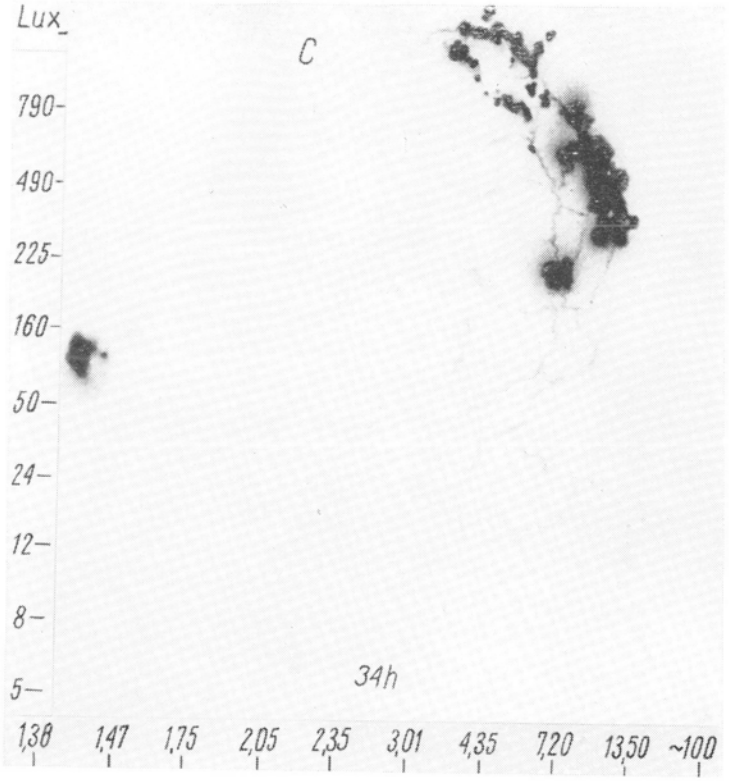

Fig. 9. Fructification of plasmodia within the crossed gradients of light and humidity

A — after 6 hours; *B* — after 24 hours; *C* — after 34 hours