

## Studies on pigments of the myxomycete *Physarum nudum*

### II. Separation and optical properties of the pigments from plasmodia cultured in darkness

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#### Abstract

The paper presents the data on separation and some optical properties of the pigments obtained from the plasmodium of the myxomycete *Physarum nudum* grown in the dark. Pigment separation was performed by means of thin-layer chromatography with cellulose MN 300 as adsorbent and with the solvent: tert.-butylalcohol, H<sub>2</sub>O, 3N NH<sub>4</sub>OH at the ratio 5:2:1. In these conditions the chromatograms revealed 12 coloured bands from which pigments were eluted and their absorption spectra as well as the spectra of fluorescence emission were determined. The isolated pigments differ from one another by their physical properties (different  $R_f$  values, localization of absorption maxima, and behaviour in acid solution). Nevertheless, certain analogies perceptible between particular pigments permitted to distinguish 3 families of the studied pigments demonstrating similar properties.

#### INTRODUCTION

In the previous study (Rakoczy 1971) it has been described the extraction method which permits of a complete elution of pigments from a plasmodium. Besides, this method makes possible a crude separation of pigments. In this way three pigment fractions were obtained from plasmodia cultured in darkness and their absorption spectra were determined. However, a closer analysis of these spectra revealed that none of these fractions was a solution of one pigment but that it contained a more or less composite mixture of pigments.

The aim of the present investigation were the attempts at more accurate separation and more precise characteristics of some physical parameters of the pigments present in the plasmodia of the myxomycete *Physarum nudum* cultured in the dark.

## MATERIAL AND METHODS

The investigations were carried out on the myxomycete *Physarum nudum* cultured by Howard's method (1931). The experimental material consisted of the plasmodia which had been grown in a dark thermostate for 7 days.

The extraction procedure for the pigments present in the plasmodia of the myxomycete under study has been described in the previous paper (Rakoczy 1971).

In order to attain a more accurate separation and more detailed characteristics of the plasmodial pigments the method of thin-layer chromatography was used. For the chromatography cellulose MN 300 as an adsorbent were used. The plates were developed in the following solvents: tert.-butylalcohol for chromatography,  $H_2O$ , and 3N  $NH_4OH$  at the ratio 5:2:1 and 7:2:1. The chromatograms were developed at the temperature of 22–23°C. After the plates had been dried, the chromatograms were viewed in visible light and in UV. The  $R_f$  values were calculated for separate bands. The pigments contained in each band were eluted with slightly acidified methanol. The absorption of methanol solution of pigments was assessed by means of an SP B 500 recording spectrophotometer in the range of 250–700 nm. The absorption measurements were made in neutral, acid and alkaline solutions (adjusted to pH 3 on application of HCl and to pH 10 when NaOH was used). In some cases rechromatography of a given pigment was performed in the same or different solvent in order to check its purity.

The present paper does not give any data on the quantitative relations of the pigments present in plasmodia. Therefore, in order to facilitate a comparison of the character of absorption curves for particular pigments the extinction values of their spectra were converted, assuming that extinction in the maximum of near UV was equal to 1.

The spectra of fluorescence emission of separate pigments were measured by means of the apparatus described by Frackowiak and Surma (1968). The excitation wave length was 375 nm. The above spectra were standardized to 1, similarly as in the case of absorption spectra.

## RESULTS

Several attempts at separation of pigments were made with column, paper and thin-layer chromatography. With the view of this, various adsorbents and solvents were applied, some of which had been employed by different researches who studied pigments of the myxomycetes. While developing thin-layer chromatograms in the solvent described by Wolf (1959) as well as by Nair and Zabka (1966), i.e. in 3N  $NH_4OH$ , a hardly perceptible separation of pigments was yielded, and the chromatograms viewed in UV demonstrated very numerous luminous bands lying close to one another. The application of the solvent composed of n-butylalcohol,  $H_2O$  and acetic acid at the ratio 4:5:1 (Nair and Zabka 1966) brought about a migration of all pigments together with the solvent, no separation occurring. On

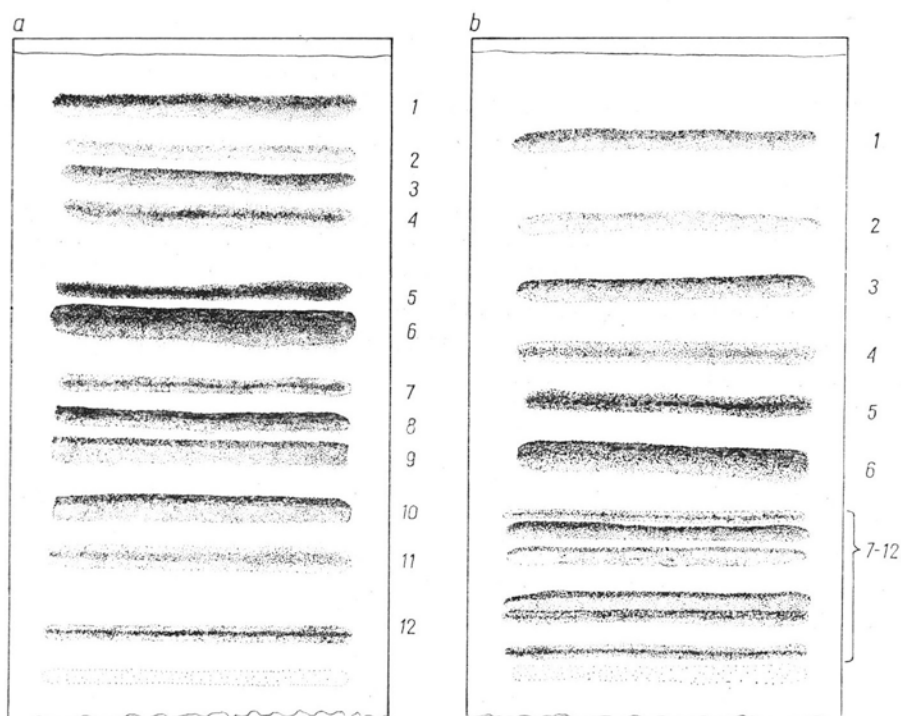


Fig. 1. Schematic representation of the separation of pigments on thin-layer chromatograms  
*a* — chromatogram developed in: methanol, water, 3N  $\text{NH}_4\text{OH}$  (5:2:1), *b* — chromatogram developed in: methanol,  $\text{H}_2\text{O}$ , 3N  $\text{NH}_4\text{OH}$  (7:2:1). Numbers at the right side show the separated pigments

the introduction of the solvent of the composition: benzine (Kp. 100–140°C), acetone and chloroform, at the ratio 5:5:4, or benzine, chloroform (100:3), and other solvents mentioned by Hager (1966) or other combinations of these compounds, all the pigments remained on their starting line. This occurred after the application of adsorbents recommended by Hager (1966) as well as on cellulose. Similar effects were produced by combinations of the above compounds with ether. The best results were obtained by using thin-layer chromatography with cellulose and the solvent: tert.-butylalcohol,  $\text{H}_2\text{O}$ , 3N  $\text{NH}_4\text{OH}$ , at the ratio 5:2:1. In these conditions 12 coloured bands were yielded from the pigments contained in fraction I. A schematic picture of the plate developed in the solvent 5:2:1 (Fig. 1a) presents the arrangement of the bands which have been numbered from 1 to 12. The chromatograms observed in visible light show 12 yellow bands of varying colour intensity. The strongest intensity of colour is manifested by bands 6, 5, 8, and 9, while that of bands 1 and 11 is the weakest. The chromatograms viewed in UV reveal band 1 as yellow-greyish, bands 2 to 6 of orange luminosity, band 7 as willow green-yellow, bands 8 and 9 as dark -green, and bands 10, 11 and 12 of lemon-yellow luminosity.

In the solvent 7:2:1, i.e. containing more butanol, the separation of pigments 1–6 which lie at some distance from the remaining pigments 7–12 is better. This solvent was used particularly to lower the  $R_f$  value of band 1, which on application of the solvent 5:2:1 migrates near the front line. Moreover, in this solvent bands 2 to 6 are more widely spaced, which ensures a better elution of them. In the solvent 7:2:1 the remaining bands (7 to 12) are poorly separated and adequate elution is difficult. Table 1 gives  $R_f$  values for individual bands and for both solvents. A schematic picture of the plate after its development in solvent 7:2:1 is presented in Fig. 1b. The absorption spectra and emission spectra of the fluorescence of separate pigments after they have been eluted are presented in Figs. 2 to 7. Besides, absorption maxima are compared in Table 1.

Table 1

$R_f$  value and absorption maxima (in near UV range) of the individual pigments

No. of pigment	$R_f$ values:		Absorption maxima in nm.	
	Solvent: butylalcohol, H <sub>2</sub> O, 3N NH <sub>4</sub> OH		pH=7	pH=3
	5:2:1	7:2:1		
1	0.92	0.86	340–345	330–360
2	0.85	0.72	340–350	(340), <u>358</u> , 378
3	0.81	0.62	340–350	(342), <u>358</u> , 378
4	0.75	0.52	344–350	(344), <u>358</u> , 378
5	0.62	0.48	346–360	(344), <u>358</u> , 378
6	0.56	0.40	346–354	(344), <u>360</u> , 378
7	0.46	0.26	372–376	380–400
8	0.42	0.22	376–380	390–410
9	0.33	0.16	380	390–410
10	0.26	0.12	390–396	400–420
11	0.20	0.1	398	410–430
12	0.07	0.03	400	404–418

Numbers in parenthesis = shoulder; underlined numbers = the main maxima.

Pigment fractions II and III demonstrate the same  $R_f$  values as corresponding bands of fraction I, and the eluted pigments possess identical optical properties with the pigments obtained from fraction I. They were omitted since the aim of the present study was the characterization of pigments and not determination of their quantitative relationships. On the other hand, the occurrence on fraction II of the bands corresponding to bands 8 and 9 of fraction I (trace band 7), and the appearance of the bands corresponding to bands 10 and 11 (trace 9) from fraction I after separation of fraction III, indicate that pigments marked with these numbers are harder to elute from a plasmodium with methanol alone. Part of these pigments (a small one) persists in the sediment left over after extraction with methanol and in order to elute them completely it is indispensable to apply 10% trichloroacetic acid (TCA). Unlike these, bands 2–6 are eluted completely with methanol.

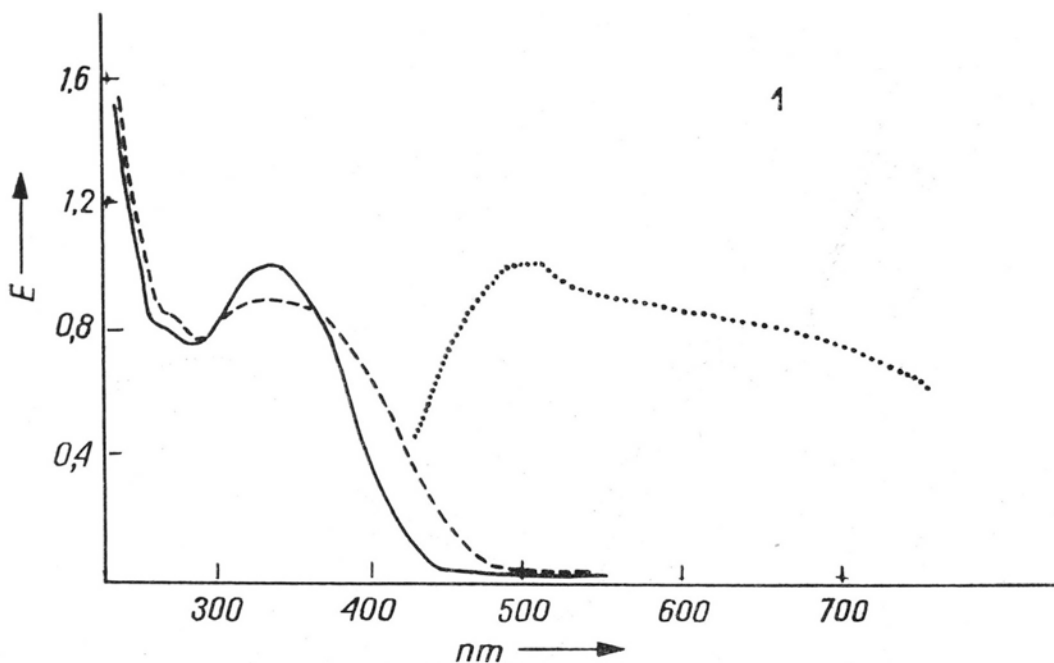


Fig. 2. Spectral characteristic of the pigment No. 1

x - axis: wavelength in nm, y - axis: extinction. Continuous line - absorption spectrum in methanol, pH = 7, extinction value for near UV peak is taken as 1.0; broken line - absorption spectrum of the same pigment concentration in methanol, pH = 3; dotted line - emission spectrum of the fluorescence (in neutral). The maximum is taken as 1.0.

The analysis of the absorption spectra reveals that the separated pigments fall into some groups with similar properties. On this basis the existence of 3 pigment families can be suggested. The first one include but one pigment No. 1, which is characterized by high  $R_f$  value and broadened maximum after acidifying. The second family contains pigments 2 to 6. All of them demonstrate a broad maximum in a neutral solution and fluorescence maxima ranging from 650 to 700 nm. Acidifying brings about a remarkable change of absorption spectrum. There appear sharp absorption maxima whose extinction value is higher in comparison with that at pH 7, more or less distinct shoulders on the short-wave side of the maximum, and a shoulder or a peak on the long-wave side of the main maximum. The increase of extinction in the maximum after acidifying to pH 3 is progressive for pigments 2 to 6. These relationships are illustrated in Fig. 7. The pigments 7 to 12 can be regarded as the third family. All of them demonstrate sharper than in the former group maxima at pH 7 and bathochromic shifts of the maximum after acidifying (about 20 nm). In the alkaline solution (pH 10) all pigments show a shift maxima towards the short-wave side of the spectrum. The maximum of the fluorescence spectrum emission is broad within the range of 500 to near 600 nm. It should be emphasized that the efficiency of the fluorescence of this group of pigments is about 10 times lower than of the pigments of the 2nd group.

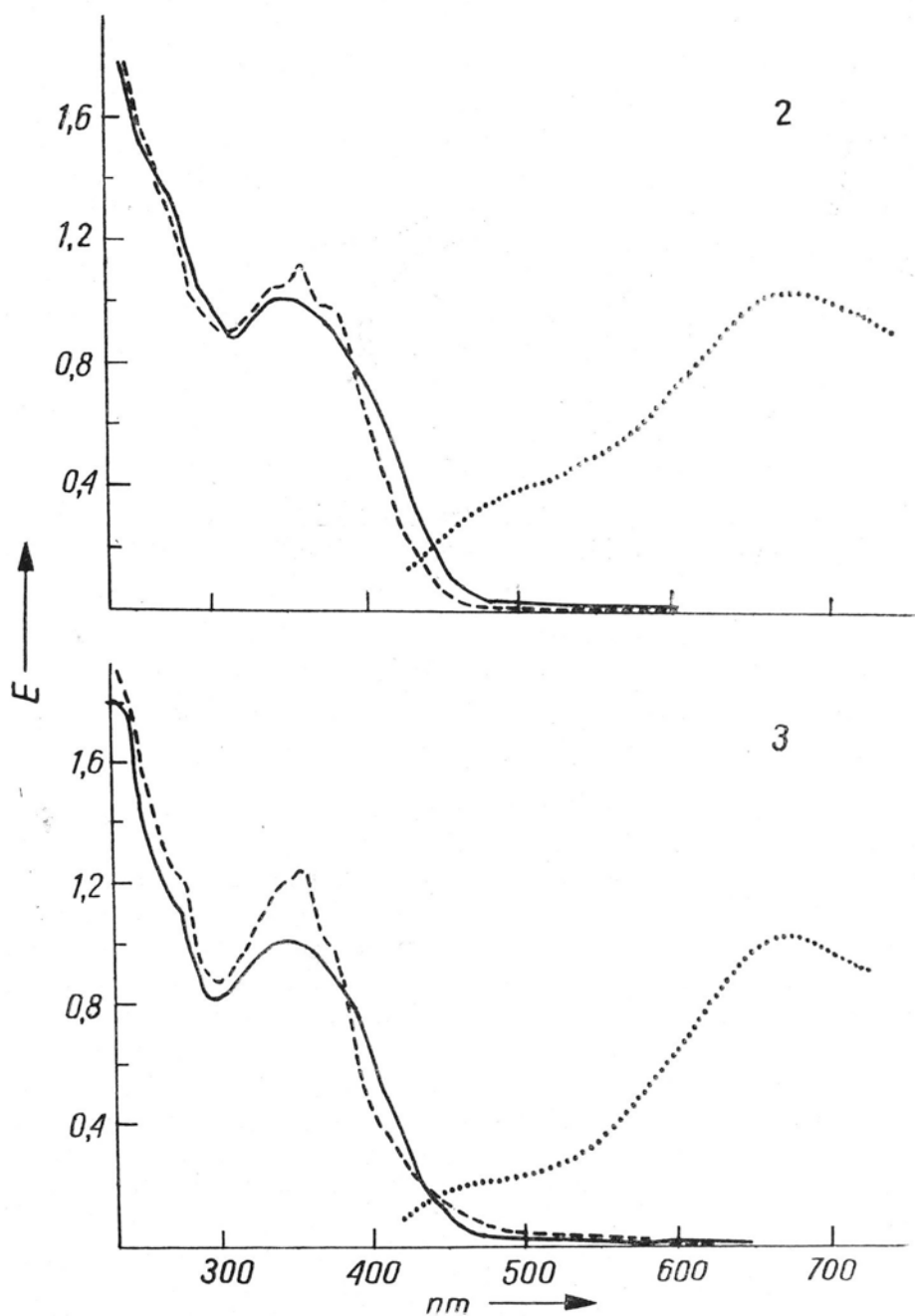


Fig. 3. Spectral characteristic of the pigments No. 2 and 3. Details as on Fig. 2.

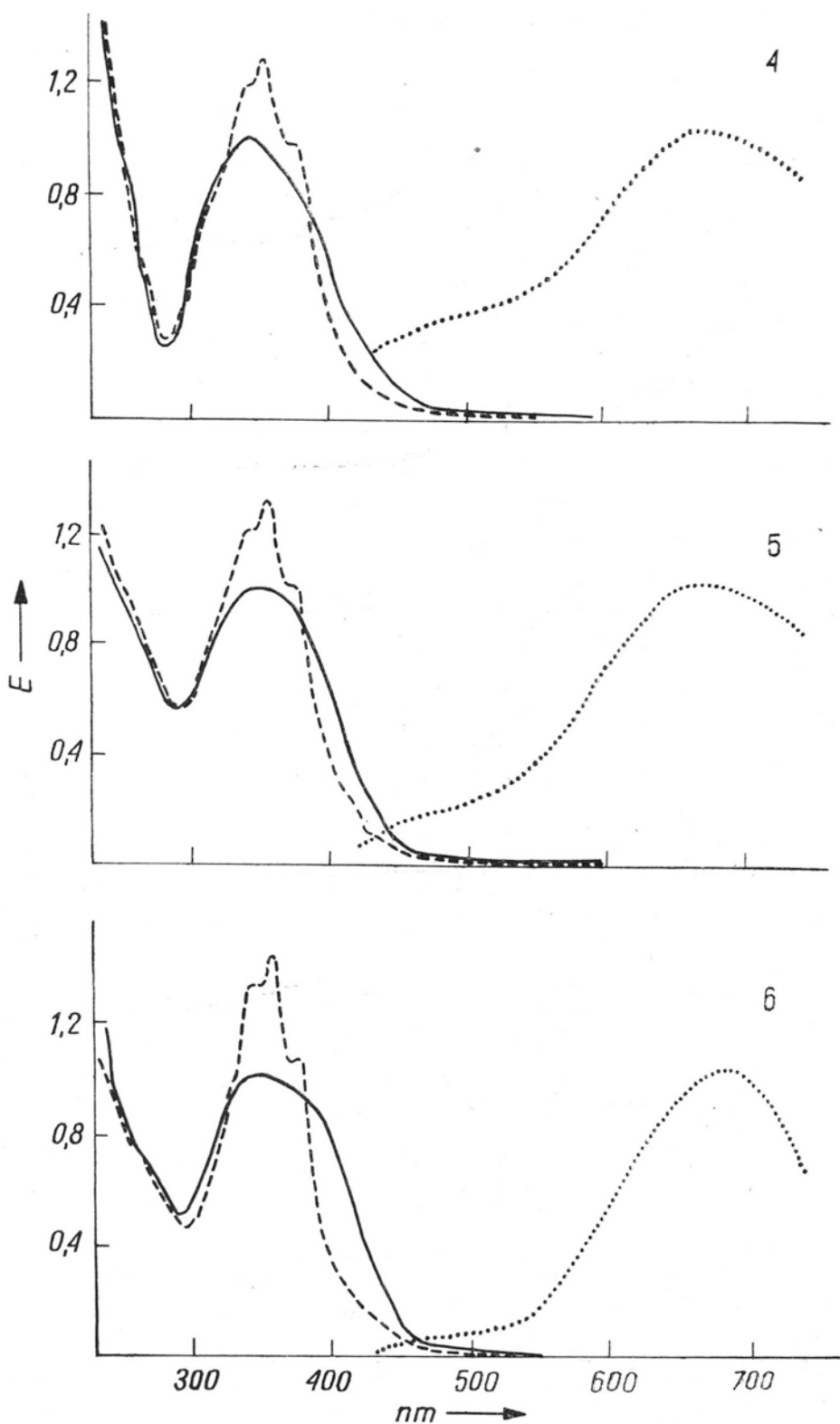


Fig. 4. Spectral characteristic of the pigments No. 4, 5 and 6. Explanation as on Fig. 2

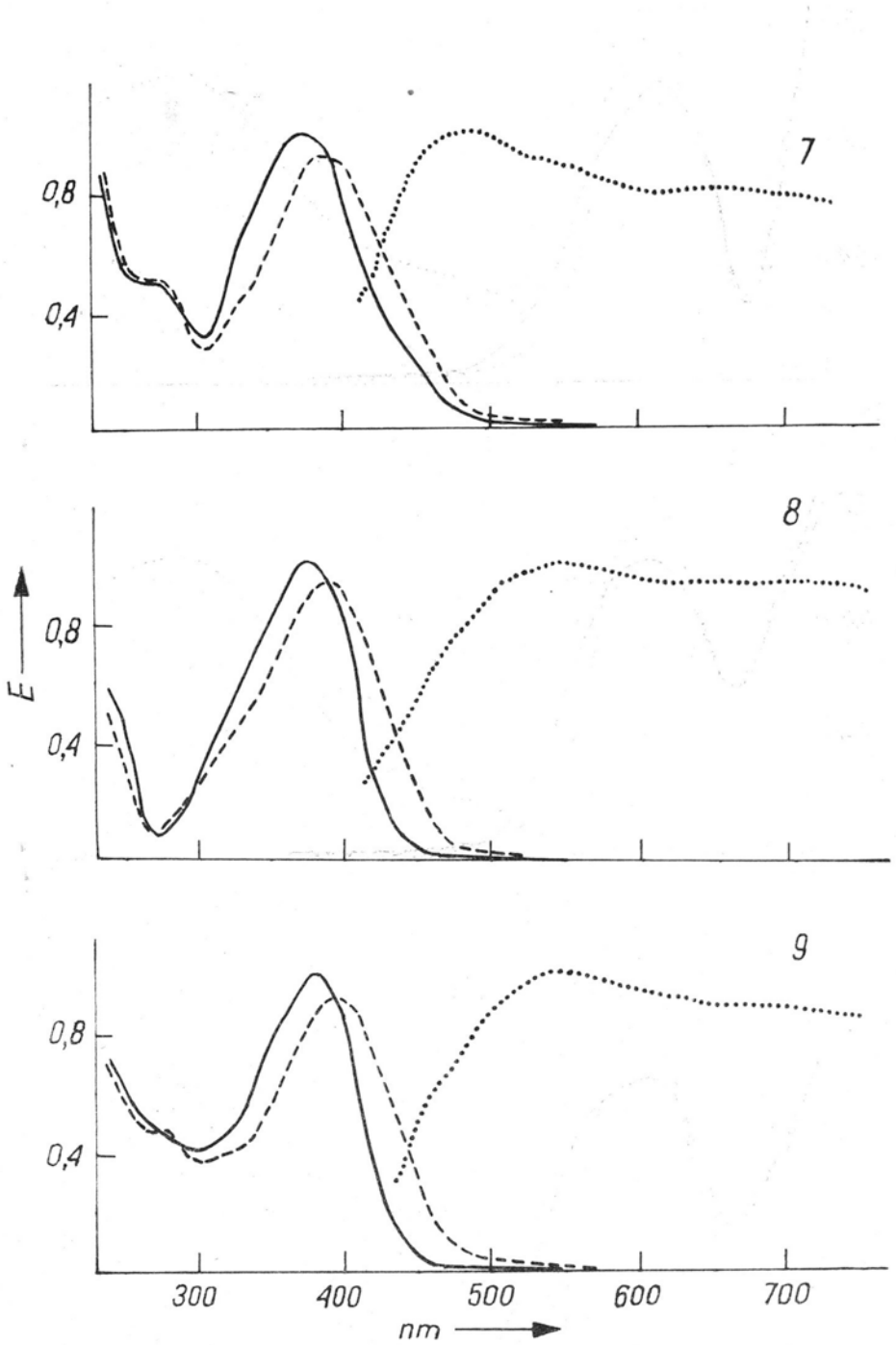


Fig. 5. Spectral characteristic of the pigments No: 7, 8 and 9. Explanation as on Fig 2.



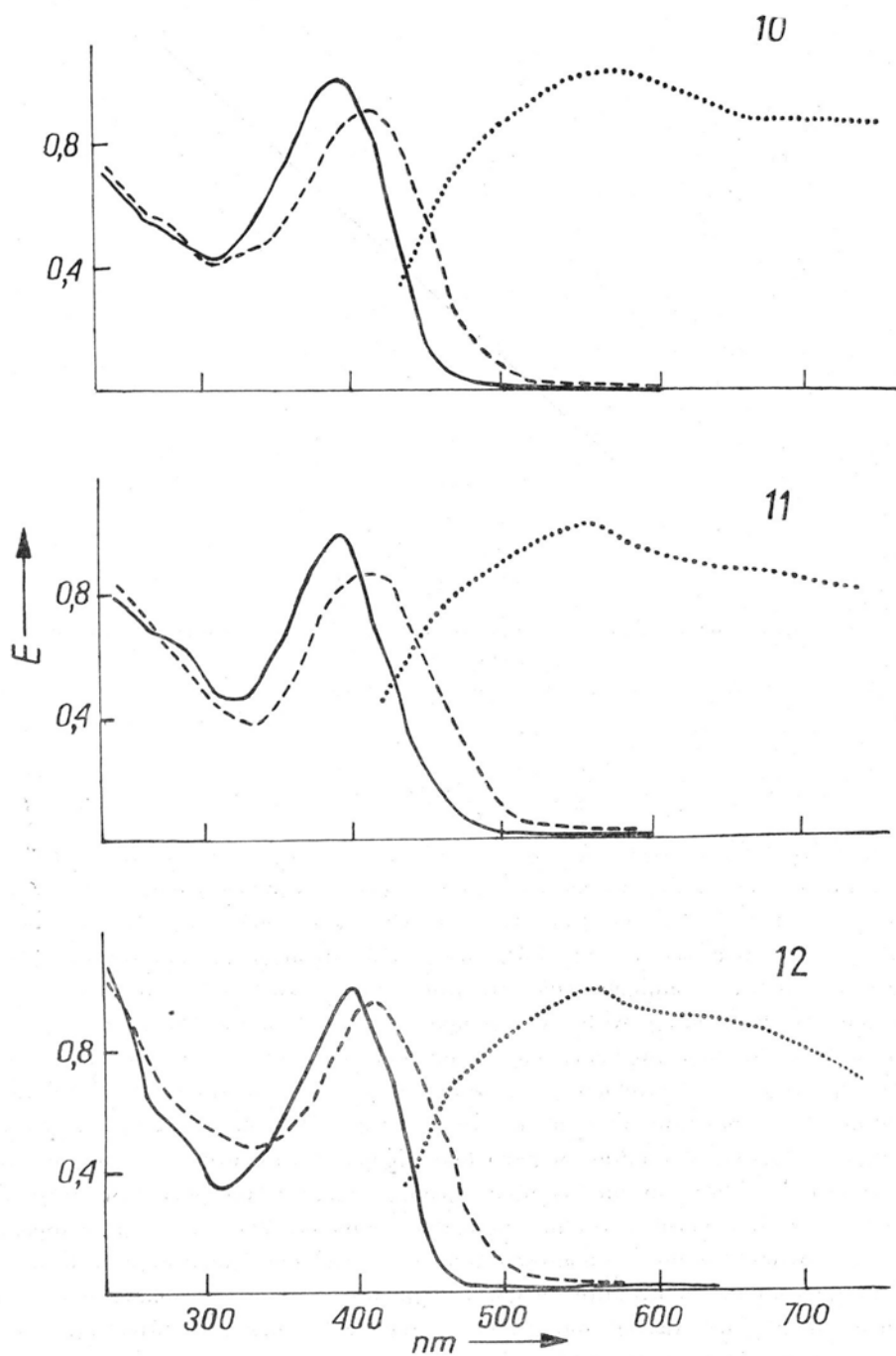


Fig. 6. Spectral characteristic of the pigments No. 10, 11 and 12. Explanation as on Fig. 2.

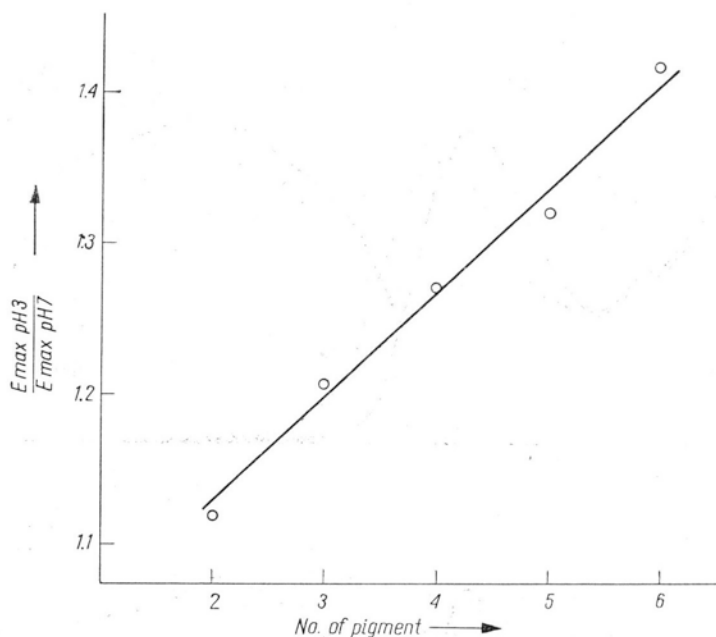


Fig. 7. Relative increase of the near UV peaks of the pigments No. 2-6 after acidification of the methanol solution.

x - axis: number of pigment, y - axis: ratio of the extinction maximum at pH = 3 to the extinction maximum at pH =

## DISCUSSION

Methanol, which was applied for the first stage of pigment extraction, has certain protein-denaturing properties. Nevertheless, a complete pigment extraction from plasmodia is achieved after treatment with TCA. There is a question if the effect of TCA consists in something more than stronger protein denaturation. However, this seems unlikely, since fractions II and III contain pigments which are qualitatively identical with some pigments of fraction I. This means that no qualitative variation of pigments that would point to a different character of TCA effect, e.g. to a rise of artefacts, is encountered in these two fractions. A different quantitative composition of pigments in fractions I, II, and III seems to testify to different degrees of binding of particular pigments with proteins. It is only the application of a more specific agent of stronger denaturing effects on proteins, such as TCA, that permits to elute pigments completely. Pigments 1 to 6 appears to be less strongly bound with protein and are completely eluted even with no or but partial protein denaturation, whereas pigments 7 to 12 presumably enter into stronger bonds with protein and become completely eluted only after have been entirely separated from proteins.

Moreover, the author checked the possibility of pigments extraction from the plasmodium of the studied myxomycete with 80% aqueous acetone solution (simi-

larly as was the case with pigments of the myxomycete *Physarum polycephalum*, Brewer, 1965). All the same, this procedure does not ensure a complete pigment elution and it is only after treatment with TCA that the rest of pigments in the sediment are eluted.

By employment of the above described method of pigment separation 12 markedly differing bands (in respect of  $R_f$  values, and optical properties) were obtained with thin-layer chromatography. This is the largest amount of pigments yielded by the plasmodium of a one species of a myxomycete. Wolf (1959), while applying paper and column chromatography and separation in 3 N  $\text{NH}_4\text{OH}$ , received 2 pigment components from the plasmodium of *Physarum polycephalum*, whereas Nair and Zabka (1966), using the same solvent for paper chromatography obtained 6 bands for the same species of a myxomycete. On the employment of the solvent: n-butanol,  $\text{H}_2\text{O}$ , acetic acid the latter investigators found 5 fractions for *Physarum polycephalum*, only 3 of them being discernible in visible light. Dresden (1959) applied paper chromatography in the solvent: methanol, benzene, butanol, water (20:10:10:11.1) obtained 4 colour bands (which were well separated) of the crude purified A-2 pigments from plasmodia of *Physarum polycephalum*. By chemical fractioning methods Brewer (1965) received 3 pigments from plasmodia of *Physarum polycephalum*, one of which seems to be an artefact. Lieth (1954), employing electrophoresis obtained 4 coloured components from the plasmodia of *Didymium nigripes*.

A comparison of the optical properties of the obtained pigments with the literature data is far from being easy because only some authors mentioned the absorption spectra of the obtained pigments. The species *Physarum polycephalum* is most closely related with *Physarum nudum* so it seems that the present results can, to some extent be compared rather with the findings for *Physarum polycephalum*. Component I described by Wolf (1959) demonstrates affinity with the family of pigments which include pigments 7 to 12 on account of absorption spectrum and bathochromic shift of the absorption maximum in acid solution. Some pigments described by Dresden (e.g. A-3), resemble in their absorption spectra (in methanol solution, neutral as well as acidified) the bands 10, 11 and 12 obtained in the present investigation. The pigment denominated A by Brewer (1965) reveals in 80% of aqueous acetone solution has a similar character of absorption as bands 2 to 6 for *Physarum nudum* in methanol solution after acidifying, whereas pigment B is characterized by a bathochromic shift following acidification, similarly as pigments 7 to 12 described in the present paper.

It is difficult to establish at the present moment whether all obtained pigments occur in the plasmodium in vivo. The distinguishing of the above families seems to be justified, and a supposition may be put forward that different pigments of the same family show a similar elementary structure and differ in but tiny details of their structure, degree of polymerization, etc.

As it has been demonstrated in investigations on the pigments of the myxomycete *Physarum nudum* grown in the light (Rakoczy 1971), the composition of pigments

in the illuminated plasmodia undergoes essential changes, particular pigment families behaving in a different way.

Neither can the chemical nature of isolated pigments be ascertained now. Studies on identification of these compounds are under way.

#### ACKNOWLEDGEMENT

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#### *Badania nad barwnikami śluzowca Physarum nudum*

##### *II. Separacja i własności pigmentów pochodzących z plazmodiów hodowanych w ciemności*

##### Streszczenie

Praca zawiera dane dotyczące rozdziału i niektórych własności fizycznych barwników uzyskanych z plazmodiów śluzowca *Physarum nudum* hodowanych w warunkach ciemności.

Separację pigmentów przeprowadzono metodą chromatografii cienkowarstwowej z celulozą MN 300 jako adsorbentem i solventem: III-rz butanol do chromatografii, woda, 3N  $\text{NH}_4\text{OH}$  w stosunku 5:2:1. W tych warunkach na chromatogramach uzyskiwano 12 barwnych warstw, z których eluowano barwniki i określano ich widma absorbcyjne i widma emisji fluorescencji.

Każdy z wyizolowanych barwników różni się od pozostałych własnościami fizycznymi (różne  $R_f$ , położenie maksimum absorpcji, zachowanie się w środowisku kwaśnym). Istnieją jednak pewne analogie między poszczególnymi barwnikami, które pozwoliły na wyróżnienie 3 rodzin pigmentów o podobnych własnościach.