FOLIA PARASITOLOGICA (PRAHA) 20: 97—103, 1973.

ISOLATION OF LIMAX AMOEBAE FROM THE NASAL MUCOSA OF MAN

L. ČERVA, C. SERBUS and V. SKOČIL

Army Institute of Hygiene, Epidemiology and Microbiology, Prague

Abstract. We examined 3,082 smears of nasal mucosa obtained from 1,551 recruits of a North Bohemian district, in culture on agar plates with a heat-killed suspension of *Aerobacter aerogenes*. Limax amoebae were found in 7 % of the samples; most frequent were *Acanthamoeba polyphaga* (5.3 %), *A. castellanii* (0.67 %) and *Hartmannella vermiformis* (0.58 %); the remaining amoeba species occurred only occasionally. The species composition was similar in both the soldiers inspected and in the dust samples collected from the soldiers' dormitories. The presence of amoebae in the mucosa of the upper respiratory system of man may be ascribed to a passive contamination of the mucosa by cysts aspirated from the external environment; a similar mode of contamination may occur with cysts of pathogenic amoebae of the genus *Naegleria*.

During the course of epidemiological studies in military communities (Skočil et al. 1970 a, b; 1971) we recovered a large number of amoeba strains from culture of smears from the nasal mucosa. Information on the composition of species in these isolates ranges among the most important data to be obtained from any general epidemiological evaluation. Our part of the study had to be published in a separate paper, i. e., much later than the statistical evaluation of the numerical results and other epidemiological observations in view of the fact that it is difficult to assess the identity of the various limax amoebae, that this work requires a lot of time and often, the results are problematic. Our study is part of a comprehensive research assignment concerned with investigations of the epidemiology and ecology of the causal microorganism of amoebic meningoencephalitis and of other closely related microorganisms.

MATERIAL AND METHODS

Our studies were conducted with amoebae obtained by culture of smears from the nasal mucosa of 1,551 recruits aged 20—21 years. These were divided into five experimental groups: a selected team (1,000 soldiers); Group 1 (150 soldiers); Group 2 (115 soldiers); Group 3 (125 soldiers); Group 4 (161 soldiers). Except the selected team, all soldiers were stationed permanently in barracks in a North Bohemian district. Members of Group 1—4 were examined 4—6 times. Our study lasted from 1968—1971.

Collection of amoebae: Most strains were obtained from the nasal mucosa. We used sterile cotton wool on wooden skewers moistened with sterile distilled water, applying one swab to both nostrils. In addition we collected 4 times dust samples from the 6 dormitories of Group 4 by skimming the surface of the beds and of other furniture with moistened cotton wool swab. From these samples we isolated 248 strains capable of growing at 37 °C, which are included in our study.

Culture method: The cotton wool swab with the collected material was broken off the skewer by means of sterile pincers and pressed into the agar layer covered with a film of a heat-killed suspension of *Aerobacter aerogenes* (Culbertson et al. 1965). The incubated plates were placed in polyethylene bags, closed hermetically and incubated upside down at 37 °C for a minimum of 7 days. In the microscope we inspected the culture through the bottom of the petri dish (magnification 100).

The isolates were passaged in the same medium. A small number of isolates only could be transferred into liquid medium (2 % Bacto Casitone Difco in distilled water) under axenic conditions.

Cloning: In view of the large number of isolates, cloning was performed only in cases in which the different shape of the amoebae and their cysts suggested the presence of two or more species in one agar plate. In most cases, however, the culture was morphologically homogeneous. The method employed for the isolation of the clones has been described in an earlier paper (Červa 1971).

Staining methods: Permanent slides were stained with Heidenhain's iron haematoxylin, Masson's green trichrome, and with Giemsa—Robinow's method for nucleic acids.

Identification of the amoebae: The criteria given by Page (1967a, b, 1968) were used for basic classification and assessment of the taxonomic position, thereby keeping in mind that often the border line between individual species is problematic owing to the vast range of variability in the shape and size of these protozoans. Unfortunately, no more satisfactory criteria are available as yet.

Photographs: Viable amoebae and cysts were photographed in the phase contrast with Heine's condenser (Ortholux Leitz, Wetzlar), at a lower magnification directly through the bottom of the petri dish and the agar layer; photographs of cysts with an immersion objective were taken in a drop of water on the microscopic slide.

RESULTS

Of a total of 1,551 soldiers examined, positive culture results were obtained from 185 soldiers, i.e. 11.9 %. We isolated a total of 218 strains of limax amoebae growing at 37 °C in the agar. We included only strains capable of growing at man's body temperature and thus fulfilling the primary condition of parasitism.

Table 1 shows the frequency of the individual species in the various groups examined.

Table 1. General survey of findings of individual amoeba species in the groups examined*)

	Group					Total	% in the no. of examinations
	selected	1	2	3	4		
No. of members in the group	1,000	150	115	125	161	1,551	
No. of cultures	1,065	729	386	551	310	3,041	
Of these positive:							
Acanthamoeba astronyxis	1	0	0	0	1	2	0.06
Acanthamoeba castellannii	4	0	13	2	2	21	0.67
Acanthamoeba polyphaga	46	38	30	35	16	165	5.3
Hartmannella exudans	1	1	0	0	0	2	0.06
Hartmannella vermiformis	4	2	1	5	6	18	0.58
Vahlkampfia jugosa	1	0	0	1	0	2	0.06
Vahlkampfia inornata	1	0	0	0	0	1	0.03
Unidentified strains	0	0	2	2	3	7	0.22
Total	58	41	46	45	28	218	7.0

*) See p. 97

Table 2. Positive findings of amoebae in repeated collection from Groups 1—4*)

	Group 1							Group 2					Group 3						Group 4				
	collection							collection					collection						collection				
	1	2	3	4	5	6	total	1	2	3	4	total	1	2	3	4	5	total	1	2	3	4	total
Acanthamoeba astronyxis	—	—	—	—	—	—	0	—	—	—	—	0	—	—	—	—	—	0	—	—	—	1	1
Acanthamoeba castellanii	—	—	—	—	—	—	0	7	4	1	1	13	1	1	—	—	—	2	1	—	—	1	2
Acanthamoeba polyphaga	1	8	4	7	12	6	38	7	13	4	6	30	9	6	6	10	4	35	5	5	1	5	16
Hartmannella exudans	—	1	—	—	—	—	1	—	—	—	—	0	—	—	—	—	—	0	—	—	—	—	0
Hartmannella vermiformis	—	—	—	—	1	1	2	1	—	—	—	1	2	2	1	—	—	5	—	—	1	5	6
Vahlkampfia jugosa	—	—	—	—	—	—	0	—	—	—	—	0	1	—	—	—	—	1	—	—	—	—	0
Unidentified strains	—	—	—	—	—	—	0	1	—	1	—	2	2	—	—	—	—	2	1	—	—	2	3
Total no. of positive findings	1	9	4	7	13	7	41	16	17	6	7	46	15	9	7	10	4	45	7	5	2	14	28
%	0.6	6.9	3.3	5.3	11.8	8.7	5.6	14.5	17.0	7.7	7.4	12	13.5	8.1	7	9	3.6	8.1	5.3	3.8	2.7	18.4	9.0
No. of examined persons	150	131	125	128	112	83	729	115	100	77	94	386	115	108	102	115	109	551	131	129	74	76	310
Month of collection	Oct.	Nov.	Jan.	Feb.	Apr.	May		May	July	Sept.	Oct.		Oct.	Nov.	Dec.	Jan.	Feb.		April	April	May	June	

*) See p. 97.

It gives evidence of the supreme dominance of *Acanthamoeba polyphaga* (75 %). The incidence of *Acanthamoeba castellanii* (9.5 %) and *Hartmannella vermiformis* (8 %) was considerably lower. Although the remaining species occurred only sporadically, a certain importance is ascribed to their presence in view of the considerably wide spectrum of isolated species. This indicates, theoretically, that any species of limax amoebae may come into contact with the nasal cavities of man. We were mildly surprized by the absence of *Naegleria gruberi* in our isolates. The idenfication of this species is not difficult and it seems hardly possible that we could have mistaken it for any other species of the family Vahlkampfiidae.

The results of culture of Group 2 disclosed a more frequent incidence of *Acanthamoeba castellanii* than that in the other groups. Although unable to give a unanimous explanation of this fact it is possible that the dominance of this species was of local and temporary nature. Table 2 illustrating variation in the species composition of the individual mucosa samples obtained from Group 1—4 shows that the incidence of *Acanthamoeba castellanii* in Group 2 was restricted to samples collected in May and July.

The culture results of samples obtained from the remaining groups did not disclose any strict dependence of qualitative findings on either the season or month of collection. The quantitative percentile findings, however, disclosed a cummulation of lower values in the winter months. Findings were most numerous in samples collected in the late spring and in the summer. The distribution of these data is shown in Fig. 1.

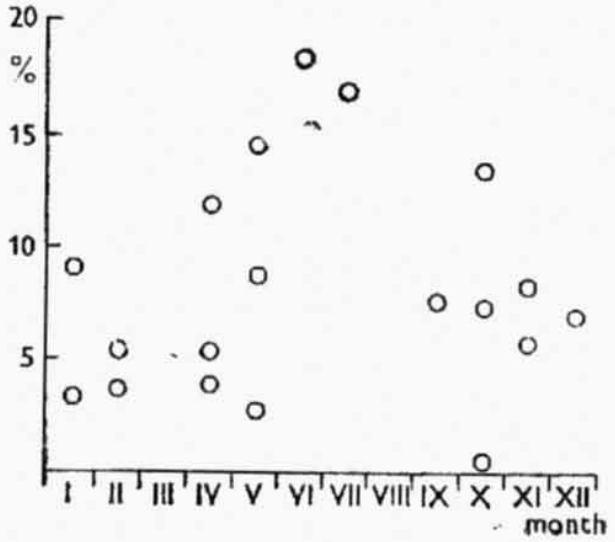


Table 3. Findings of amoebae in dust samples from the dormitories of group 4*)

Species	Collection				Total	%
	1	2	3	4		
Acanthamoeba castellanii	7	3	0	6	16	3.4
Acanthamoeba polyphaga	53	54	32	24	163	34.6
Hartmannella exudans	0	0	1	1	2	0.42
Hartmannella vermiformis	4	5	4	21	34	7.2
Unidentified species	2	1	3	1	7	1.4
Total no. of culture samples	150	150	85	92	477	
Of these positive	64	59	37	52	212	45.1

*) See p. 97

Repeated positive findings in samples from a single person were not exceptional. Although the same amoeba species was found in samples from the same person sometimes two or three times, the intervals being as long as several months, this is no proof as yet of potential carriership in view of the absolute dominance of *Acanthamoeba polyphaga* in samples of nasal mucosa. Only in one case, another species, i.e., *Hartmannella vermiformis*, was found repeatedly.

Table 3 surveys the amoeba species obtained from the external environment of Group 4. It shows that the species composition is very similar to that in Table 1 giving evidence of the close association between the composition of amoeba species isolated from the external environment of the persons examined and that from nasal cavities. It seems very probable that the external environment is responsible for the contamination of the nasal cavities and, therefore, may be considered to be the natural source of infection.

DISCUSSION

The finding of limax amoebae in smears or rinses from nasal and pharyngeal mucosa referred to in the papers by several authors (Wang and Feldman 1961, 1967; Pereira et al. 1966, Armstrong et al. 1967, Warhurst et al. 1967) were mostly incidental, because the initial purpose of these studies was the isolation of viruses from the upper respiratory tract. Only Shumaker et al. (1971) performed their experiments with the intention of disclosing the presence of amoebae in culture of nasal smears in agar and in tissue culture. They examined 155 children before and after bathing in lakes in the vicinity of Richmond. They isolated a strain of *Naegleria gruberi* from the nasal mucosa of a 7 year—old boy taken immediately after the boy had returned from his swim. The strain growing at 24 °C was evidently not pathogenic. The experiments indicate that contamination of the nasal mucosa from an aqueous enviroment is possible, which is an important observation from the epidemiological point of view.

The method of isolation employed in our experiments utilizes agar plates with *Aerobacter*, nowadays recognized as the most effective culture medium. The critical point of this method is the migration of amoebae from the cotton wool tampon to the agar, because the sites of direct contact are very small. On the other hand, considerably larger numbers of amoebae are obtained with a tampon than with rinses or scraping of the mucosa. Until a more effective method will be available, we shall have to put up with this methodological insufficiency.

Originally we assumed that the species determination of isolates from the nasal mucosa of man will complete essentially the quantitative results of statistical and epidemiological evaluation obtained earlier from other sections of this investigation. These results, however, did not offer the expected elucidation of the problem, whether the positive findings in man were the consequence of an even temporary stage of carriership, during which multiplication of the vegetative amoeba stages may have occurred on the surface or in the mucosa of the upper respiratory tract, or whether infection was acquired from cysts inhaled with dust.

In view of the dominant incidence of *Acanthamoeba polyphaga* only, the distribution of the individual species in our material does not offer any proof of an active carriership of limax amoebae in man. On the other hand, the marked similarity in the composition of species isolated from Group 4 and from their dormitories indicates a passive contamination of the nasal mucosa. It is of interest that an infusorian of the order Spirotricha was found growing in one of the agar plates inoculated with material from the nasal mucosa.

The identification of species disclosing a marked similarity in the species composition of material isolated from the soldiers and in that obtained from their external environment indicated the possibility of acute danger of aerogenic transmission of resistant stages of pathogenic amoeba strains, e.g. *Naegleria* sp. This mode of transmission is suggested by the presence of amoebic cysts related to the genus *Vahlkampfia*, which possesses similar biological properties. The absence of *Naegleria* sp. in our isolates may be explained by the local composition of the soil biocenosis.

The most frequent species isolated from both the nasal mucosa and the external environment was *Acanthamoeba polyphaga*. The cyst of this species is covered with a multiply layered wall and this accounts, apparently, for the high resistance to desiccation and its prolonged survival in a dry environment. Also this species is one of the potential pathogenic agents in spite of the fact that no records are available as yet on infection of man with *A. polyphaga*. The dominance of *Acanthamoeba* in our material is in accord with the findings of Kingston and Warhurst (1969) obtained from the air, although their designation of the species is different from ours.

The increased frequency of positive findings during the warm seasons is very logical as regards the increased amount of dust in the air. Repeated positive findings in the same individual, and the earlier observed and statistically distinct relationship between pathological changes of the nasal mucosa and the frequency of amoebae in the culture may, in this connection, be explained by the fact that the natural self-cleaning activity of the nasal mucosa (ciliated epithelium) may be reduced and retarded in an inflamed or otherwise affected mucosa. Under these circumstances it is very probable that cysts will be isolated from the mucosa. Contact of a pathogenic strain with such suitable environment increases the possibility of excystation and of active attachment of vegetative stages and, consequently, the development of acute disease.

ВЫДЕЛЕНИЕ АМЕБ ТИПА „LIMAX" ИЗ НОСОВОЙ СЛИЗИСТОЙ ОБОЛОЧКИ ЧЕЛОВЕКА

Л. Черва, Ц. Сербус и В. Скочил

Резюме. Мы исследовали 3.082 мазков из носовой слизистой оболочки, полученных от 1.551 новобранцев из одного северо-чешского района, в культуре на агаре с суспензией убитых жаром *Aerobacter aerogenes*. Амеб типа „limax" находили в 7 % проб; чаще всего встречались *Acanthamoeba polyphaga* (5,3 %), *A. castellanii* (0,67 %) и *Hartmannella vermiformis* (0,58 %); остальные виды амеб встречались лишь случайно. Видовой состав оказался одинаков как у обследованных солдат так в пробах пыли, собранной из солдатских общежитий. Наличие амеб в слизистой оболочке верхней дыхательной системы человека можно приписывать пассивной контаминации слизистой оболочки цистами, попавшими туда при дыхании из окружающей среды; подобным способом может произойти поражение цистами патогенных амеб из рода *Naegleria*.

REFERENCES

ARMSTRONG J. A., PEREIRA M. S., Identification of "Ryan Virus" as an amoeba of the genus *Hartmannella*. Brit. Med. J. 1: 212—214, 1967.

ČERVA L., Studies of limax amoebae in a swimming pool. Hydrobiologia (Haag) 38: 141—161, 1971.

CULBERTSON C. G., ENSMINGER P. W., OVERTON W. M., The isolation of additional strains of pathogenic *Hartmannella* sp. (*Acanthamoeba*). Proposed culture method for application to biological material. Am. J. Clin. Pathol. 43: 383—387, 1965.

KINGSTON D., WARHURST D. C., Isolation of amoebae from the air. J. Med. Microbiol. 2: 27—36, 1969.
PAGE F. C., Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. J. Protozool. 14: 499—511, 1967 a.
—, Re-definition of the genus *Acanthamoeba* with descriptions of 3 species. J. Protozool. 14: 709—724, 1967 b.
—, Generic criteria for *Flabellula*, *Rugipes* and *Hyalodiscus*, with descriptions of species. J. Protozool. 15: 9—26, 1968.
PEREIRA M. S., MARSDEN H. B., CORBITT G., TOBIN J. O'H., Ryan virus: a possible new human pathogen. Brit. Med. J. 1: 130—132, 1966.
SHUMAKER J. B., HEALY G. R., ENGLISH D., SCHULZ M., PAGE F., *Naegleria gruberi:* isolation from nasal swab of a healthy individual. Lancet 7724: 602, 1971.
SKOČIL V., ČERVA L., SERBUS C., Epidemiological study of amoebas of the limax group in military communities. First report: J. Hyg. Epid. Microbiol. Immunol. 14: 61—66, 1970.
—, —, —, NEJEDLO V., Epidemiological study of amoebas of the limax group in military communities. II. Study of the military community L. I (1968—1969). J. Hyg. Epid. Microbiol. Immunol. 14: 324—331, 1970.
—, SERBUS C., ČERVA L., Epidemiological study of the incidence of amoebas of the limax group in military communities. III. Investigation of the community of 3rd garrison L I.—Problems of contagion in the community. J. Hyg. Epid. Microbiol. Immunol. 15: 156—162, 1971.
WANG S. S., FELDMAN H. A., Occurrence of acanthamoebae in tissue cultures inoculated with human pharyngeal swabs. Antimicrobial Agents & Chemotherapy 1: 50—53, 1961.
—, —, Isolation of *Hartmannella* species from human throats. New England J. Med. 277: 1174—1179, 1967.
WARHURST D. C., ARMSTRONG J. A., Study of a small amoeba from mammalian cell cultures infected with Ryan virus. J. gen. Microbiol. 50: 207—215, 1968.

Received 25 January 1972.

L. Č., Vojenský ústav hygieny, epidemiologie a mikrobiologie, U vojenské nemocnice 1200, Praha 6, ČSSR

FOLIA PARASITOLOGICA: (PRAHA) 20: 103—104, 1973

CASES OF FACULTATIVE PARASITISM OF LYCTOCORINAE (HETEROPTERA: ANTHOCORIDAE) ON MAN IN CZECHOSLOVAKIA

Apart from the family Cimicidae the cases of Heteroptera biting man in Central Europe may be roughly classified in three groups.

A) Large predatory species of aquatic families (Notonectidae, Naucoridae, Aphelochiridae, Nepidae—rarely) and of terrestrial Reduviidae can painfully pierce human skin when incautiously handled; even spontaneous attacks by *Ilyocoris cimicoides* (L.) and *Reduvius personatus* (L.) may occur and doubtful cases of attempts at blood-sucking were reported for the latter species. B) Numerous species of both predatory and phytophagous terrestrial families (mainly Nabidae, Miridae and Anthocoridae: Anthocorinae) may accidentally and spontaneously attempt to pierce human skin and even actually suck lymph or blood, particularly in hot weather on perspiring individuals. These cases are of little theoretical interest, since probably any sufficiently active species capable of fast piercing action may become occasionally involved. The resulting annoyance is usually negligible, but that caused by normally acarophagous and aphidophagous nymphs and adults of *Orius* spp. (Anthocorinae) during hop-picking is probably the most severe. C) More theoretically and practically important are the cases of true facultative ectoparasitism of nymphs and adults of Anthocoridae: Lyctocorinae, since in this subfamily numerous morphological and physiological characters and behavioural traits evolved which parallel those of Cimicidae and which are sometimes regarded as evidence of their closest cladistic relationship. *Lyctocoris campestris* (F., 1794) is the only Central European species important in this respect. It is a general predator feeding mainly on small arthropods in nests of a variety of birds, but living also subcortically and occurring in various synanthropic habitats as well. Under not yet elucidated conditions it occasionally

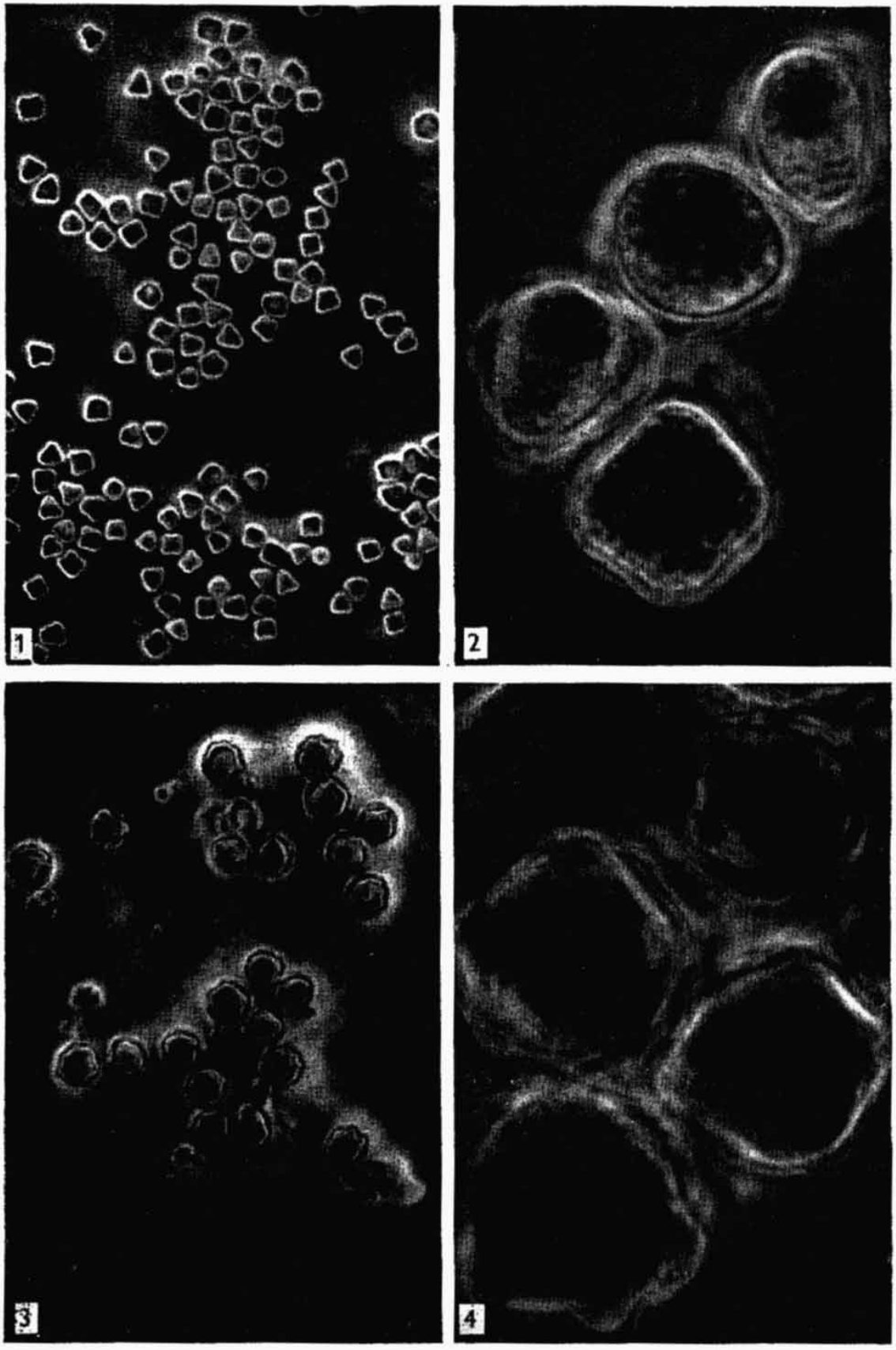


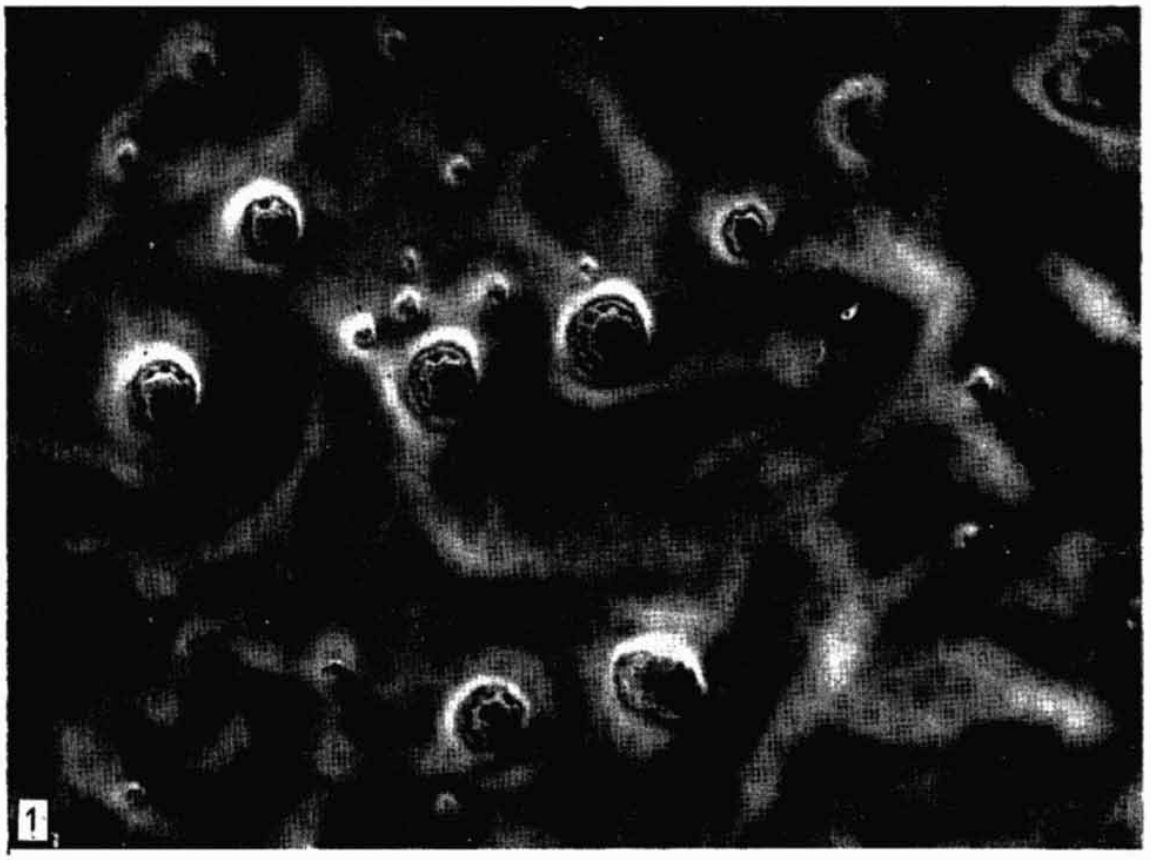
Plate I

Fig. 1. *Acanthamoeba polyphaga.* Cysts on the surface of the agar plate. Triangular till quadrangular forms are dominating. Phase contrast (×300).

Fig. 2. *Acanthamoeba polyphaga*, showing the multilayered wall of the cyst with the escaping pores. Phase contrast (×2,000).

Fig. 3. *Acanthamoeba castellanii.* Cysts on the surface of the agar plate. Note the characteristic polygonal till spherical shape. Phase contrast. (×300).

Fig. 4. *Acanthamoeba castellanii.* Cysts with a multilayered, irregularly folded, wall. Focussing was difficult in view of the spherical shape. Phase contrast (×2,000).



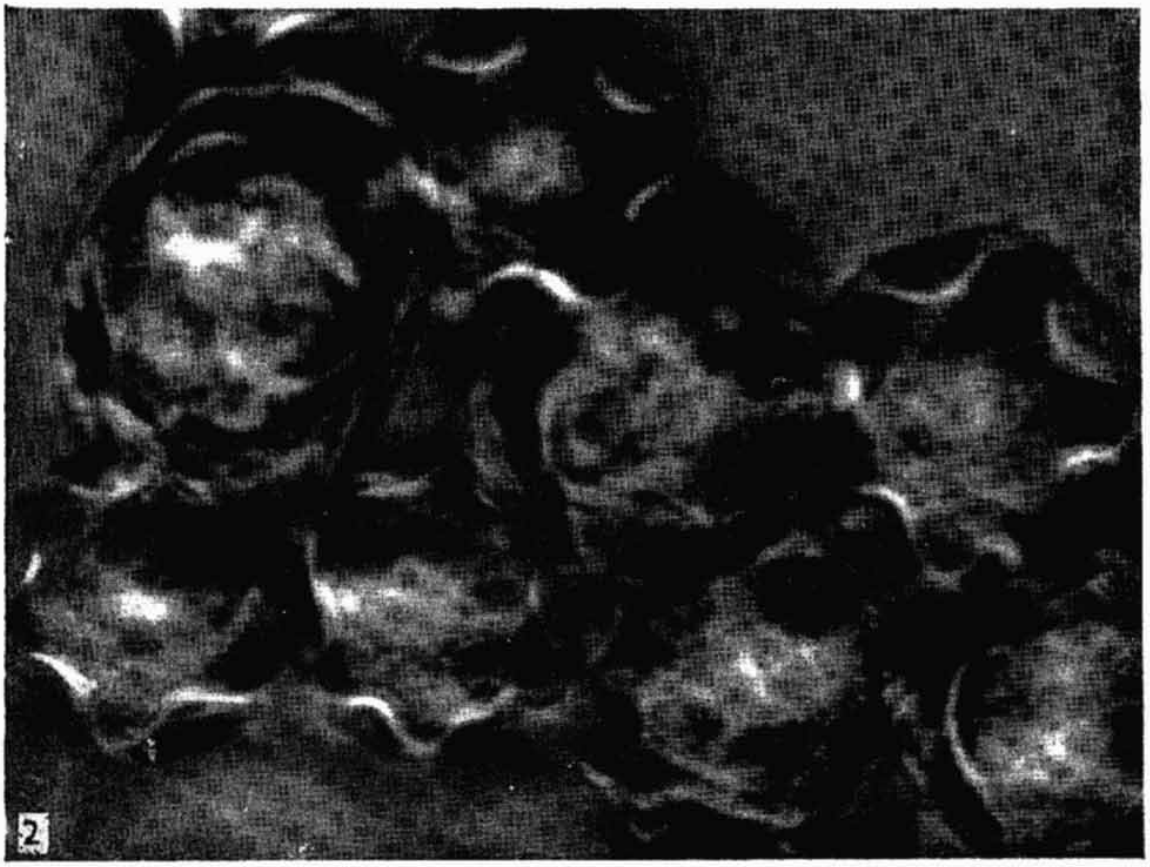


Plate II

Fig. 1. *Acanthamoeba astronyxis.* Cysts on the surface of the agar plate. Phase contrast (×300).
Fig. 2. *Acanthamoeba astronyxis.* Endocyst of regular starlike shape. Slanting cysts disclose their lens-shaped flattening. Phase contrast. (×2,000).

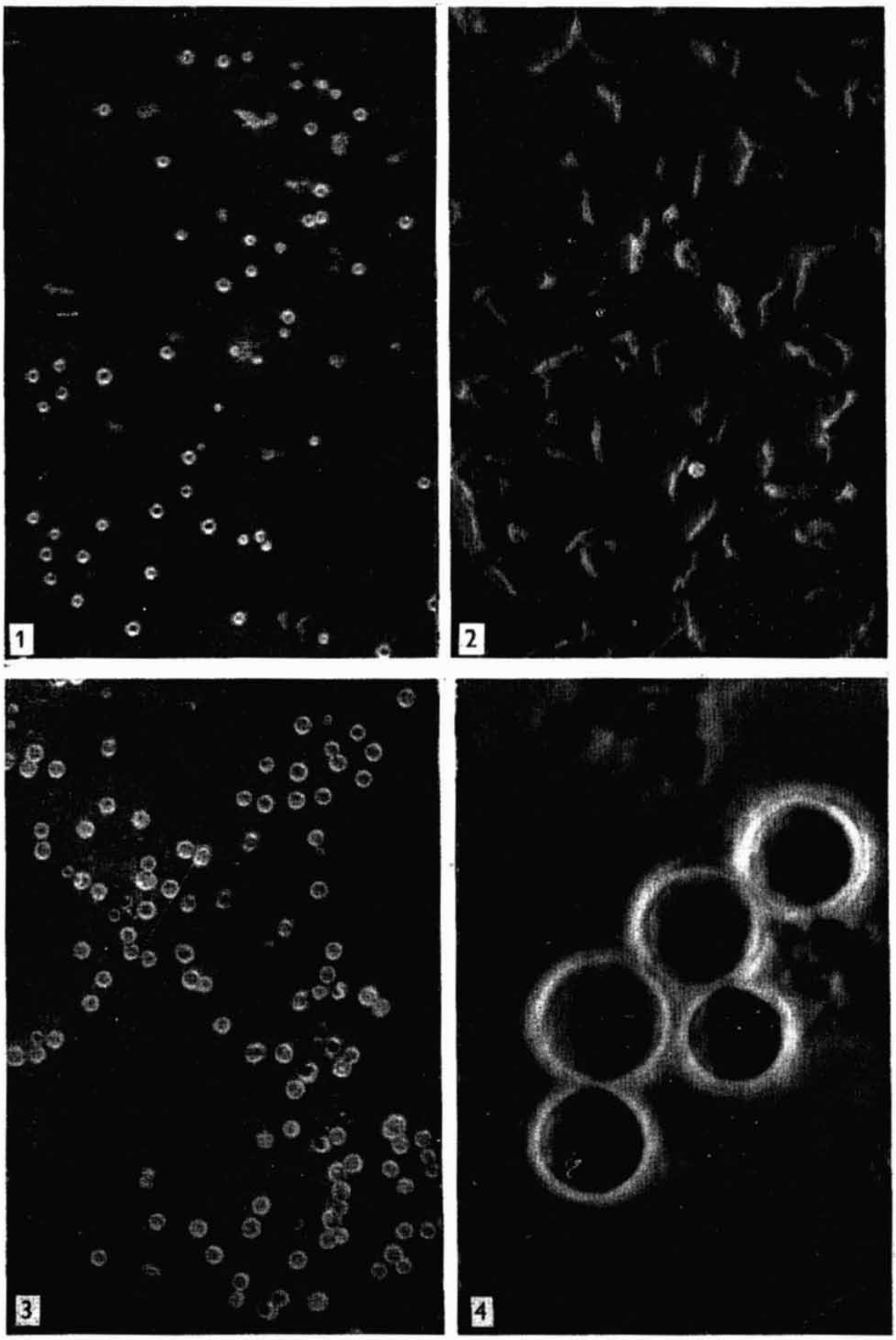


Plate III

Figs. 1. 2. *Hartmannella exudans.* Cysts and vegetative stages on the surface of the agar plate. Phase contrast (×300).

Fig. 3. *Hartmannella vermiformis.* Cysts on the surface of the agar plate. Phase contrast (×300).

Fig. 4. *Hartmannella vermiformis.* Spherical cysts with almost smoothly contoured walls. Phase contrast (×2,000).

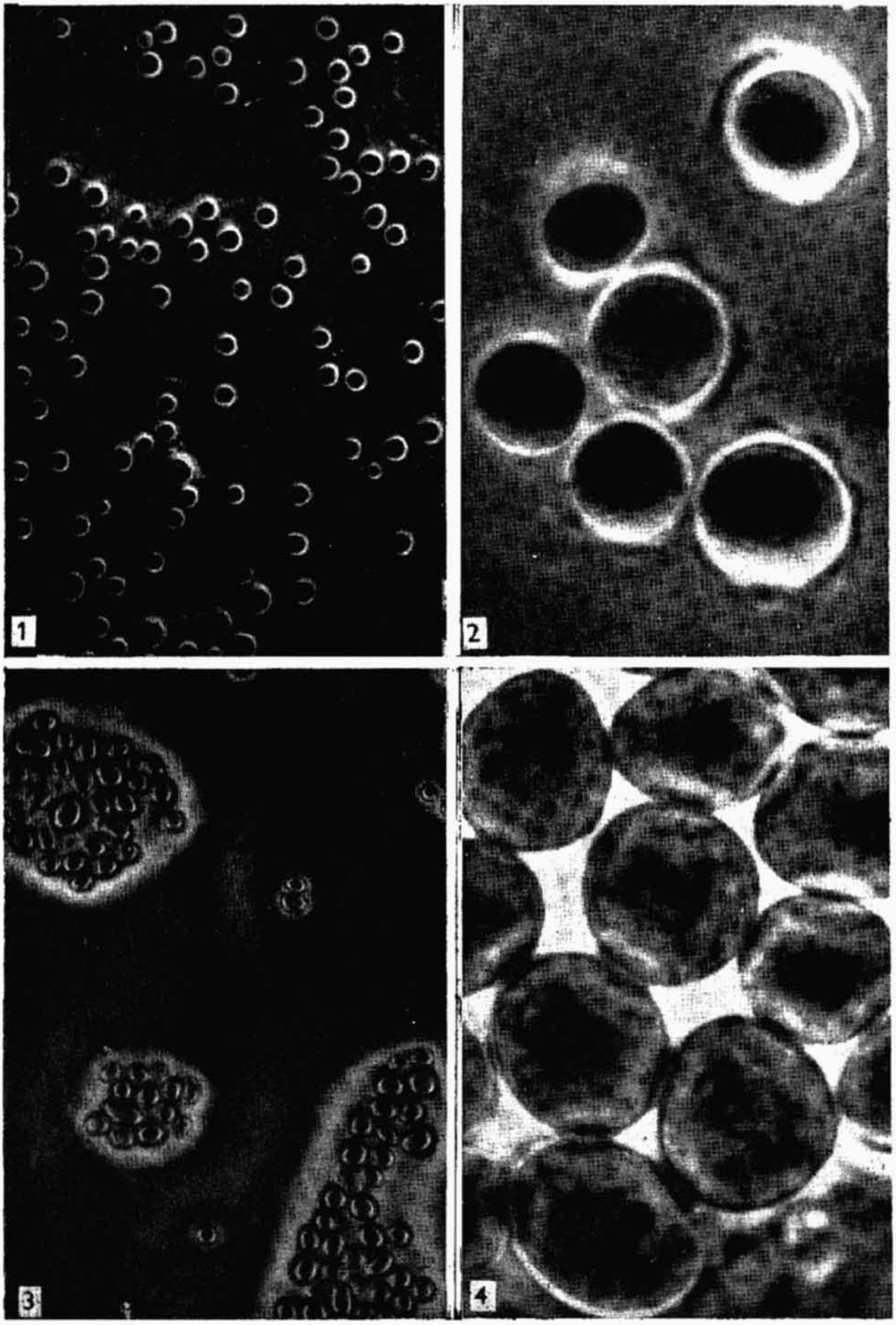


Plate IV

Fig. 1. *Vahlkampfia jugosa*. General view on the cysts on the surface of the agar plate. Phase contrast (× 300).

Fig. 2. *Vahlkampfia jugosa*. Undulated contours of the mucous exocyst seen only in the phase contrast (× 2,000).

Fig. 3. *Vahlkampfia inornata*. Cysts on the surface of the agar plate. The grouping of cysts is typical of amoebae of the family Vahlkampfia. The lens-shaped cyst becomes visible through the marked reflection of its margin. Phase contrast (× 300).

Fig. 4. *Vahlkampfia inornata*. The wall of the cyst is relatively thin and smooth without visible escape pores. The nucleus and nucleolus are clearly visible. Phase contrast (× 2,000).