THE UPTAKE OF FLUORESCENT ALBUMIN BY PINOCYTOSIS IN *Amoeba proteus*

CICILY CHAPMAN-ANDRESEN and HOWARD HOLTZER. From the Physiological Department of the Carlsberg Laboratory, Copenhagen, Denmark. Dr. Holtzer is a Scholar in Cancer Research of the American Cancer Society, Inc.

Holter and Marshall (7) demonstrated the uptake of fluorescein-labelled globulin by pinocytosis in the large ameba *Chaos chaos* (*Pelomyxa carolinensis*) and followed the fate of the ingested material in the cell by measurement of fluorescence and by observations on centrifuged amebae; their uptake experiments were made at pH 6.8. Brandt (1), also using fluorescein-labelled globulin at a similar pH, in the same species, showed that the plasmalemma and the walls of the pinocytotic vacuoles adsorbed the protein; he confirmed these results with specific antibody tests. Marshall, Schumaker, and Brandt (11) have discussed the importance of binding of protein to the surface of cells engaged in pinocytosis. A review by Holter (9) summarizes recent advances in this field. That many different types of protein induce pinocytosis has been shown by Chapman-Andresen and Prescott (5), and the effect of the pH of albumin solutions on the intensity of pinocytosis in *Amoeba proteus*, as determined by the number of channels observed, has been demonstrated by Chapman-Andresen (3); in aqueous solutions of bovine plasma albumin, pinocytosis is very intense at pH 4.1 to 4.8, while at pH 5.5 or above, it is completely absent.

It was thought worthwhile to check the effects of pH on pinocytosis in amebae using fluorescein-labelled albumin. The amoebae were immersed in fluorescent protein solutions at acid and at neutral pH, and by employing different conditions for washing the amebae after removal from the fluorescent protein solution, attempts were made to investigate the conditions for the binding of the protein to plasmalemma.

The experiments reported here were designed to explore these possibilities in a qualititive manner; quantitative experiments involving counting of pinocytosis channels and measurements of fluorescence in groups of amebae have been planned and are in progress.

Supported in part by a grant from The Rockefeller Foundation, and in part by Research Grants B-493 and B-1629 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service.
Received for publication, May 9, 1960.

MATERIALS AND METHODS

Amebae were cultured according to Prescott (12), but in a modified Pringsheim's solution (2), fed on *Tetrahymena* (Cophenhagen strain), and starved for 2 days before use. The fluorescent protein was prepared by labelling bovine plasma albumin (Armour and Co. crystalline) with fluorescein after the manner of Coons (6), and was dialysed against Pringsheim's solution for 2 days before use. The total protein concentration was 0.7 per cent; the pH was adjusted to 4.2–4.5 ("acid") (found to be the optimal pH for pinocytosis channel formation (3)) or to 6.5–6.9 ("neutral") (no pinocytosis channels are seen at this reaction (3)) by the addition of N/10 HCl or NaOH. All pH measurements were made with a glass electrode pH meter (Radiometer, pH M22, Copenhagen).

The amebae were immersed for 30 minutes in the fluorescent protein solution, and then washed, by transferring with a braking pipette (8), through a series of 5 salt cellars, each of which contained one of the following solutions: (1) 0.7 per cent non-fluorescent bovine plasma albumin at pH 4.2–4.5 ("acid protein wash"); (2) as (1) but at pH 6.5–6.9 ("neutral protein wash"); (3) Pringsheim's solution at pH 4.2–4.5 ("acid Pringsheim wash"); (4) as (3) but at pH 6.5–6.9 ("neutral Pringsheim wash"). The total washing time was about 5 minutes, and the total dilution factor, from the fluorescent protein solution to the final (5th) washing solution, was of the order of $1:10^{10}$.

The amebae were observed, either living, or fixed in alcohol or in formalin, in a standard Zeiss fluorescence microscope fitted with a cardioid condensor, an Osram mercury arc lamp, and the appropriate filters. Observations were made immediately after transfer to the last washing solution, and at intervals up to *ca.* 18 hours, during which time the amebae were kept in the washing solutions. At this time all amoebae were transferred to neutral Pringsheim's solution, as the acid solutions were toxic if the amoebae remained in them for longer periods. Observations were continued on these amebae up to 7 days after immersion, during which period the amebae were starved, and transferred daily to fresh Pringsheim's solution.

RESULTS

Observations on Living Amebae

Table I summarises the observations on living amebae, up to 24 hours after removal from the fluorescein-labelled albumin, as regards the presence of fluorescent vacuoles within the amebae and of fluorescent coating of the plasmalemma. The reaction of the washing solution was decisive for the preservation of membrane adsorption; the "neutral" solutions removed all traces of fluorescence from the plasmalemma during the 5 minutes washing period, while washing in "acid" solutions resulted in retention of a fluorescent coating. The intensity of this coating was greatly reduced by keeping the amebae for 30 minutes in

TABLE I

Effect of pH of Fluorescent Protein and of Washing Solution on the Presence of Fluorescent Vacuoles and of Fluorescent Surface Coating. Observations on Living Amoeba proteus

Immersion solution	Washing solution		Interval between transfer from fluorescent protein solution and observation							
			1 to 5 min.		30 min.		14 hrs.		24 hrs.	
Fluorescein-labelled bovine plasma albumin Reaction	Reaction	Composition	Fluorescein vacuoles	Surface fluorescein	Fluorescein vacuoles	Surface fluorescein	Fluorescein vacuoles	Surface fluorescein	Fluorescein vacuoles	Surface fluorescein
Acid (pH 4.2–4.5)	Acid	Protein	+	+	+	sl.	+	−	+*	−*
		Pringsheim	+	+	+	sl.	+	−	+*	−*
Acid (pH 4.2–4.5)	Neutral	Protein	+	−	+	−	+	−	+	−
		Pringsheim	+	−	+	−	+	−	+	−
Neutral (pH 6.5–6.9)	Acid	Protein	−	+	−	sl.	−	−	−*	−*
		Pringsheim	−	+	−	sl.	−	−	−*	−*
Neutral (pH 6.5–6.9)	Neutral	Protein	−	−	−	−	−	−	−	−
		Pringsheim	−	−	−	−	−	−	−	−

* Amebae transferred to neutral solutions at 18 hours.

the "acid" solutions. The composition (protein or Pringsheim's solution) of the washing medium seemed to be unimportant. Amebae which were immersed in acid-labelled protein, washed, and then immediately examined under the fluorescence microscope while still living, invariably exhibited large numbers of small fluorescent vacuoles.

Amebae, immersed in "neutral" fluorescent protein solution, and examined immediately after washing in "neutral" solution, exhibited no detectable fluorescence either on the surface or in the cytoplasm, while amebae immersed in "neutral" fluorescent protein and washed in "acid" solution showed surface fluorescence, which was still visible but fainter, 30 minutes after removal; no fluorescence was visible 14 hours after removal. 24 hours after removal from "acid" fluorescent protein solution, the amebae contained some large (*ca.* 10 μ diameter) vacuoles, which were first observed *ca.* 14 hours after immersion. These large vacuoles persisted in the starving amebae up to 7 days after immersion, when 1 to 4 palely fluorescent vacuoles with a solid fluorescent core were visible in each ameba. The small vacuoles had disappeared by 4 days after immersion; a few amebae were completely free from fluorescence by 6 days after immersion.

Observations on Fixed Amebae

Fixation in 96 per cent ethyl alcohol proved to be unsatisfactory, as diffuse fluorescence was observed to spread through the cytoplasm, regardless of the time interval between fixation and removal from the last washing solution. However, when amebae were fixed for 1 hour in 1 per cent formaldehyde in Pringsheim's solution, within 20 minutes of removal from the fluorescent protein solution, the distribution of fluorescence was identical with that seen in the living amebae, and storage of the fixed amebae for 4 days in the cold did not result in any change. However, when amebae were fixed in formalin 24 hours or more after removal from the fluorescent protein solution, the fixation resulted in considerable decrease of fluorescence; when some fluorescence remained, it was usually present in the cytoplasm, as well as in the vacuoles.

CONCLUSIONS

1. The intense formation of pinocytosis channels previously (3) observed in "acid" (pH 4.2–4.5) solutions of bovine plasma albumin was accompained by the uptake of considerable amounts of protein, as shown by the presence of many fluorescent vacuoles, while in more neutral solutions (pH 6.5–6.9) uptake was very slight or absent.

2. Washing of the amebae in "acid" solutions, whether of protein or of Pringsheim's solution, after removal from the fluorescent protein solution, resulted in the retention of a fluorescent coating on the plasmalemma for at least 30 minutes, while washing in "neutral" solutions removed all protein from the plasmalemma. Surface binding of the protein was promoted by an acid reaction. The efficiency of neutral washing solutions for removing protein bound to the cell surface has also been reported for tissue cells (10).

3. Fluorescence is gradually lost from the amebae during the period of observation (7 days); the path of this loss is not known, but it can be thought to be either by defecation of the whole content of the large fluorescent vacuoles, or by excretion by way of the contractile vacuole, although it has not so far been possible to identify a fluorescent contractile vacuole.

4. Fixation in 96 per cent ethyl alcohol results in rapid loss of fluorescence from the pinocytotic vacuoles. One per cent formalin retains the fluorescent material in the vacuoles, when fixation is made soon after the removal of the amoebae from the fluorescent protein solution, while fixation 24 hours or later results in loss of fluorescent material.

It may be suggested that this loss can be attributed either to a change in the permeability of the walls of the pinocytosis vacuoles, similar to that observed by Chapman-Andresen and Holter (4) in experiments with ^{14}C glucose in the ameba *Chaos chaos*, or to an enzymatic breakdown of the fluorescent albumin into smaller molecules, which cannot be fixed by formalin, as can the original protein. The latter possibility appears to be most likely, as fluorescence was never seen in the cytoplasm of living amebae; rupture of amebae during observation in the fluorescence microscope immediately resulted in fluorescent staining of the cytoplasm, indicating that in the living ameba the vacuole membrane is very impermeable to the, possibly degraded, fluorescent material.

We wish to express our best thanks to Professor H. Holter for his interest and advice during the course of this work.

REFERENCES

1. BRANDT, P. W., *Exp. Cell Research*, 1958, **15,** 300.
2. CHAPMAN-ANDRESEN, C., *Compt. rend. trav. Lab. Carlsberg, Sér. chim.*, 1958, **31,** 77.
3. CHAPMAN-ANDRESEN, C., paper presented at the 112th Conference of the Society for Experimental Biology, Copenhagen, 1959, unpublished.
4. CHAPMAN-ANDRESEN, C., and HOLTER, H., *Exp. Cell Research*, 1955, suppl. 3, 52.
5. CHAPMAN-ANDRESEN, C., and PRESCOTT, D. M., *Compt. rend. trav. Lab. Carlsberg*, 1956, **30,** 57.
6. COONS, A. H., and KAPLAN, M. H., *J. Exp. Med.*, 1950, **91,** 1.
7. HOLTER, H., and MARSHALL, J. M., JR., *Compt. rend. trav. Lab. Carlsberg, Sér. chim.*, 1954, **29,** 7.
8. HOLTER, H., *Compt. rend. trav. Lab. Carlsberg, Sér. chim.*, 1943, **24,** 399.
9. HOLTER, H., *Ann. New York Acad. Sc.*, 1959, **78,** 524.
10. HOLTZER, H., and HOLTZER, S., *Compt. rend. trav. Lab. Carlsberg, Sér. chim.*, 1960, **31,** 373.
11. MARSHALL, J. M., JR., SCHUMAKER, V. N., and BRANDT, P. W., *Ann. New York Acad. Sc.*, 1959, 1959, **78,** 515.
12. PRESCOTT, D. M., *Compt. rend. trav. Lab. Carlsberg, Sér. chim.*, 1956, **30,** 1.