ELECTRON MICROSCOPIC STUDIES ON THE SHAPE AND SIZE OF CALCIUM CASEINATE-PHOSPHATE PARTICLES IN MILK

By

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Introduction

Casein exists in milk as micelles or complex particles containing calcium, phosphate and others, in addition to the casein proteins. Many workers[1-8] observed the caseinate particles in milk using electron microscopes and demonstrated that they are roughly spherical ranging in diameters of less than 20 to more than 200 mμ. NITSCHMANN[1] reported actual size distribution, showing the particles with diameters of 40 to 160 mμ were most numerous. He recommended the use of 0.01 M $CaCl_2$ for dilution of the sample or fixing caseinate particles with 0.4% formaldehyde prior to dilution with distilled water. MAENO and YUSA[2] reported that casein micelles in skim milk, which was diluted 100 times with distilled water, ranged from 20 to 200 mμ in diameter. SAITO[3] demonstrated that caseinate particles ranging from 60 to 200 mμ were most numerous in skim milk diluted 400 times with distilled water. TANAHASHI[4] observed caseinate particles of normal milk and mastitis milk diluted 300 times, presenting average diameter of 121 mμ and 103 mμ respectively. HOSTETTLER[5] reported that most casein particles ranged from 10 to 200 mμ, eventhough such large particles as 800 mμ were detected.

The above mentioned workers observed caseinate particles in extremely diluted samples. A high dilution of more than 100 times may affect the size of caseinate particles more or less as mentioned by NITSCHMANN[1]. Furthermore, the single drop method, which was used extensively by other workers, permitted movement of caseinate particles when a drop of diluted sample placed on a film-covered grid was allowed to evaporate. The movement resulted in uneven distribution of caseinate particles and caused certain difficulties in estimating the size distribution of the particles.

During the course of an investigation of dissolved caseinate particles, it

[Journ. Facul., Agr., Hokkaido Univ., Sopporo, Vol. 54, Pt. 1, 1964]

became necessary to establish methods other than the single-drop method, when preparing a specimen for electron microscopic observation. The present paper presents two methods for the preparation of specimen, namely the replica method and the spray method. The methods are considered to eliminate the disadvantages of the single-drop method, namely high dilution of the sample and uneven distribution of caseinate particles. The paper also presents some examples of actual size distribution of caseinate particles.

Material and Methods

Milk: Mixed morning milk obtained from the Hokkaido University herd was used unless otherwise stated.

Individual milk was obtained from the herd at the time of evening milking. The milk was cooled in an ice-water bath within 15 minutes after milking.

In a trial, a sample of pooled milk, obtained from a storage tank of a commercial milk plant, was used.

Skimming of milk: Skim milk was prepared by centrifugation ($895 \times G$, for 10 min., at 32°C) in the same manner as another paper of authors[9].

Dialyzed sediment: Suspension of dialyzed sediment, consisting of caseinate particles, was prepared according to the author's report[9]. The sediment obtained by centrifugation ($9{,}600 \times G$, 60 min., at 4°C) of 240 ml of skim milk was suspended into 20 ml of distilled water, and followed by dialysis against distilled water for 24 hours at 4°C.

Preparation of specimen for electron microscopic observation: Preparation of film-covered grid (collodion was used as the suporting film) was made according to MAENO and YUSA's report[2]. Other common procedures, not particularly mentioned, were undertaken according to FISHER's text book[10]. The distilled water used was prepared by a glass distillator from deionized water.

(1) Replica method: A slide glass was dipped in the sample and allowed to stand vertically on filter paper. This paper absorbed the excess of sample. Thus, the slide glass was coated with a dried thin layer of the sample. The coated slide glass was then subjected to the replicating procedure. A direct-carbon replica, shadowed with chromium, was prepared. A number of washing procedures were applied to the carbon replica. The following procedure was most satisfactory to wash the replica: First the replica was floated in 1.5N NH_4OH, then in a mixture (1 : 1) of ethyl ether and ethanol, and finally in ethyl ether, each for 30 minutes in the order mentioned. Occasional agitation was helpful throughout the washing procedure. Then the washed replica was cut into small pieces. A piece of the washed replica was placed on a grid for observation.

(2) Spray method: Sample was diluted from 10- to 50-fold in volume with distilled water or some other adequate dispersion media. The diluted sample was sprayed on a film-covered grid by a plastic nebulizer, #300, manufactured by Vaponefrin Co. Polystyrene latex particles of known diameter (557 mμ, standard deviation 10.8 mμ) were mounted beforehand on the film-covered grid, as necessary, by the following procedure: The polystyrene latex, Run No. LS-063-A, No. Measurements 373, manufactured by The Dow Chemical Co., was highly diluted with distilled water prior to use. One drop of the diluted polystyrene latex sol was placed on the film-covered grid by means of a capillary pipette and allowed to evaporate. The turbidity of the diluted polystrene latex sol was measured after additional dilution (2-fold in volume) as was done with the case of skim milk. This presented an optical density reading of 0.45 to 0.50.

Shadowing: Chromium was used as the shadowing metal. Approximate two milligrams of chromium were evaporated by a tungsten filament placed 7 cm from the specimen. The angle of shadowing was 36°.

Electron microscopic observation: A Nihon Denshi Kogaku JEM-Model 5L electron microscope was used. Electron micrographs were taken at the direct magnification of 5,100-fold.

The estimation of the size of caseinate particles: Electron micrographs enlarged to a final magnification of 25,000-fold were prepared. The diameter of the caseinate particles were measured using a millimeter scale. At least 500 particles were measured. All the measurements of shadowed particles were made perpendicular to the direction of the shadow.

Measuring of the turbidity: The turbidity was determined by a photoelectric photometer, FPW-4, manufactured by Hitachi Ltd., Tokyo. The turbidity was arbitrarily defined as the reading of the optical density at the wave length of 530 mμ (No. 53 filter was used).

Determination of protein: The nitrogen concentration determind by the micro-Kjeldahl method was multiplied by 6.38.

Results

The turbidity of diluted skim milk: The skim milk was diluted to 50-fold in volume with distilled water or other types of dispersion media both with and without pretreatments. The turbidity of the diluted sample was measured after it had stood for certain periods. The results are demonstrated in Fig. 1, revealing that the turbidity decreased, in most cases, according to the proceeding of time. Diluting with 0.01 M $CaCl_2$ was the only case where

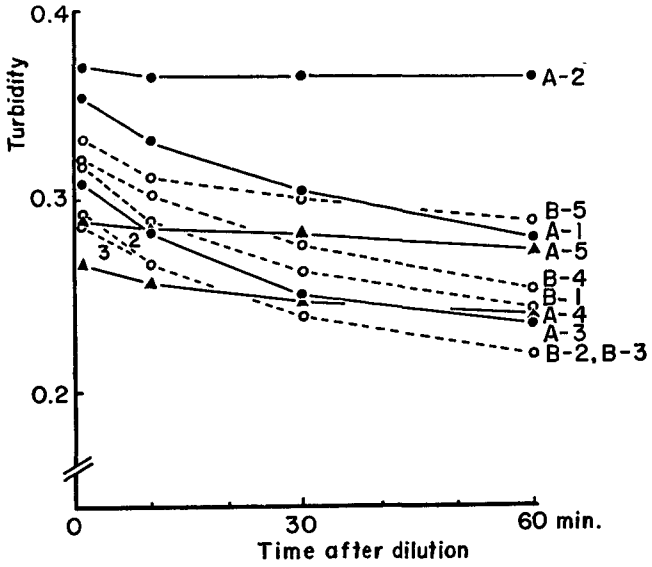

Fig. 1. Turbidity of diluted (1 : 50) skim milk. The turbidity was arbitrarily defined as the reading of optical density at the wave length of 530 mμ.

A-1: Diluted with distilled water. Protein concentration: 0.050%
A-2: Diluted with 0.01 M $CaCl_2$. Protein concentration: 0.050%
A-3: Formaldehyde was added (0.40%) prior to the dilution with distilled water. Protein concentration: 0.040%
A-4: Formaldehyde was added (0.40%), dialyzed against distilled water for 24 hours, and diluted with distilled water at 50-fold. Protein concentration: 0.036%
A-5: The same as A-4, but dialyzed against 0.4% formaldehyde. Protein concentration: 0.036%
B-1: Diluted with distilled water. Protein concentration: 0.052%
B-2: Formaldehyde was added (0.04%) prior to the dilution with distilled weter. Protein concentration: 0.046%
B-3: Formaldehyde was added (0.40%) prior to the dilution with distilled water. Protein concentration: 0.046%
B-4: Diluted with 0.04% formaldehyde. Protein concentration: 0.052%
B-5: Diluted with 0.40% formaldehyde. Protein concentration: 0.052%

the decrease of turbidity was not detected. The fixation of casein by the addition of formaldehyde at a concentration of 0.4% or 0.04%, prior to dilution with distilled water, was not effective in avoiding a decrease of turbidity. The fixation procedure also reduced the appearance of initial turbidity through the dilution effect of formaldehyde solution. The turbidity curves in Fig. 1 suggest

that preparation of a specimen should be finished within few minutes after dilution.

The observation of surface replica of caseinate particles: The replica method was applied in order to observe caseinate particles without the dilution of the samples. The surface patterns of caseinate particles, photographed from a replica of whole milk and dialyzed sediment, are given in Fig. 2, which also presented a surface replica of a fat globule. The surface of caseinate particles were coarse rather than smooth.

The observation of caseinate particles mounted by the spray method: The entire view of a droplet of diluted (1 : 50) skim milk, sprayed by the nebulizer, is presented in Fig. 3, demonstrating that caseinate particles are spherical regardless of the dispersion media. The dilution of skim milk with ultrafiltrate, prepared by the filtration of skim milk through Chamberland L3 tube, eliminated the diluting effect of distilled water. However, it was impossible to observe any caseinate particles located in the center part of the droplet because of the high density of serum components. The dilution of skim milk with distilled water caused the crowding of caseinate particles at the surface portion. This was probably due both to the rapid evaporation of water at surface of the droplet before it adhered to the collodion film and to surface tension. Parts other than the surface, however, were quite uniform in the distribution of caseinate particles, and could be used for estimation of particle-size distribution. The use of 0.01 M $CaCl_2$ as a dispersing medium was successful in not crowding the praticles at the surface portion of droplet. It is desirable that the diameter of all particles in a entire droplet are measured to estimate the size distribution of caseinate particles. It is thought, however, that the measuring of particles in a certain area of the center part of droplet, could be substituted for the measuring of all particles. This is suggested for the sake of convenience.

The determination of a correct magnification of an electron microscopy was made by using polystyrene latex particles of 557 mμ in diameter. The comparisons of polystyrene latex particles and caseinate particles in skim milk are shown in Fig. 4.

The effect of pretreatment of sample and dispersion media on size distribution of caseinate particles: Skim milk, subjected to the following pretreatments, was diluted to 50-fold with distilled water, 0.04 to 0.40% formaldehyde, or 0.01M $CaCl_2$:

1. Control (no pretreatment),
2. Fixation with 0.4% or 0.04% formaldehyde,
3. Fixation with 0.4% formaldehyde and subsequent dialysis against distilled water for 24 hours, and

4. The same as 3. but dialyzed against 0.4% formaldehyde. The size distributions of caseinate particles of the diluted sample were determined using electron microscopic patterns, which are demonstrated in Fig. 5 and 6, of Cr-shadowed specimen. The size distributions are shown in Table 1. No remarkable difference was observed in the size distribution of caseinate particles

TABLE 1. Effects of dispersion media and pretreatments on the size distribution of caseinate particles in milk.

(Frequency %)

Diameter (mμ)	Diluted with				Dialyzed and diluted with		
	Distilled water	0.01M $CaCl_2$	0.4% Formaldehyde	Distilled* water	Distilled** water	Distilled*** water	0.04%**** Formaldehyde
21 -40	21.66	10.51	20.39	23.30	26.85	22.79	28.12
41- 80	46.08	47.90	46.12	45.39	45.64	52.85	42.57
81-120	21.20	29.44	26.21	22.82	18.35	17.41	20.20
121-160	6.91	10.28	5.83	5.83	6.71	6.65	6.34
161-200	0.46	1.64	0.97	2.43	2.01	0.32	1.58
201-240	3.69	0.00	0.49	0.00	0.45	0.00	0.99
Average diameter (mμ)	88.77	89.52	79.97	76.47	74.40	73.60	76.39

* Formaldehyde was added (0.4%) prior to dilution (1 : 50).

** Formaldehyde was added (0.4%). The mixture was dialyzed against distilled water for 24 hours prior to dilution (1 : 40) with distilled water.

*** The same as ** but dialyzed against 0.4% formaldehyde.

**** The same as ** but diluted with 0.4% formaldehyde.

in various treated samples with exception of the above dilution of 0.01 M $CaCl_2$, which resulted in a slightly higher diameter average of caseinate particles than in other cases. This agrees with the turbidity curves in Fig. 1. According to the results in Table 1, dilution of skim milk to 50-fold in volume with 0.01 M $CaCl_2$ was considered to be suitable for determining the size of caseinate particles.

The size distribution of caseinate particles of individual and pooled milk: Several samples of individual milk and a pooled milk were subjected, after skimming, to the investigation of size distribution of caseinate particles by the method mentioned above. The dispersion medium used was 0.01 M $CaCl_2$. The results are presented in Table 2, showing some variations in the diameter average by individuals.

The staining of caseinate particles: Two staining reagents, phosphotungstic acid and osmic acid, were used. Staining with phosphotungstic acid was

TABLE 2. Size distribution of caseinate particles in individual milk.

Frequency (%)

Diameter (mμ)	H-1*	H-2	H-3	H-4	H-5	G-1*	G-2	G-3	G-4	G-5	Average**	Pooled *** milk
21- 40	26.25	6.96	17.91	11.48	18.84	13.86	5.94	7.32	11.72	14.38	13.47	17.89
41- 80	43.96	32.17	34.65	46.23	42.47	46.87	39.26	41.97	38.28	43.48	40.93	44.04
81-120	21.04	38.26	25.59	34.43	28.08	30.36	35.64	37.47	32.42	33.44	31.67	27.98
121-160	6.88	18.26	14.64	7.21	7.88	7.59	14.64	12.39	12.50	7.36	10.94	6.42
161-200	1.87	3.04	5.35	0.33	1.71	0.66	3.10	0.85	4.30	1.35	2.26	2.75
201-240	0.00	1.30	1.16	0.33	0.68	0.66	1.03	0.00	0.39	0.00	0.56	0.92
241-280	0.00	0.00	0.47	0.00	0.34	0.00	0.30	0.00	0.39	0.00	0.15	0.00
Average diameter (mμ)	76.70	103.26	95 11	86.18	85.30	85.62	101.06	93.72	94.24	85.69	90.69	85.32
Protein (%) ****	2.38	2.41	2.60	2.64	2.71	2.79	3.03	3.10	3.25	3.65	2.86	

* "H" stands for Holstein cow. "G" stands for Guernsey cow.

** Average of H-1 to G-5.

*** The sample was taken from 18,000 Kg of mixed milk in the storage tank of a commercial milk plant.

**** Protein concentration of skimmed portion.

achieved by diluting (1 : 50) sample (skim milk) with an aqueous 0.2% solution of the acid prior to spraying. Staining with osmic acid was done by keeping specimens in the vapor of osmic acid for 20 minutes. An airtight covered dish for diffusion analysis was used for this purpose. Dialyzed sediment was used as the sample for phosphotungstic-acid staining. Results are presented in Fig. 7, showing a stained surface layer of caseinate particles. Under these staining conditions the osmic acid was applied to skim milk, resulting in less contrast than the specimen stained with phosphotungstic acid.

Discussion

The limitation in applying electron microscopy for the investigation of biological fields is the distortion of sample due to drying. It is impossible to eliminate this drying, at present, eventhough some efforts for observation of moist specimens, enclosed in a closed chamber, are made by ABRAMS and McBAIN[11]. The distortion, however, can be minimized by using the proper method in preparing the specimen. This present study of electron microscopic patterns of milk suggests that the distortion of caseinate particles might be slight because of the simple sphere. This is case, at least, under the conditions studied. The replica of skim milk confirmed that caseinate particles are spherical as demonstrated by other workers[1-8]. Uniform distribution of caseinate particles was successfully achieved by the spray method, making it possible to estimate the correct size distribution of caeinate particles. This is done with the aid of polystyrene latex particles which have known diameter. The spray method also made possible the observation of caseinate particles in higher salt concentrations than in the single-drop method, because the amount of diluted sample deposited on the film-covered grid is so small and the evaporation of water is so rapid that the development of salt crystal is minimized or avoided. Therefore, preparation of specimen by the spray method will be extensively used in the electron microscopic studies cosncerning caseinate micelles. An example of this would be the micelle formation of casein by calcium ion. Shape, size distribution and aggregation of caseinate particles in certain salt concentrations can be suggested with high possibility by applying the spray method. The spray method is also advantageous for the time required to finish the preparation of a specimen. The turbidity study demonstrated that the turbidity of diluted skim milk reduces according to the progress of time if $CaCl_2$ was not added. This suggests the dissolving of caseinate particles to some extent. The dissolving effect may be negligible as the spray method requires only one minute or less to finish the preparation of the specimen after dilution.

The shadowing technique was extensively applied in the present study to obtain electron micrographs of better contrast. The effect of shadowing on the observed size of particles arises here for consideration. KERN and KERN[12] measured the diameter of polystyrene latex particles and demonstrated that the image size of 259 mμ changes to 284 mμ when the specimen is shadowed and that the amount of change is relatively constant. They suggested that shadowing provides an electrical contact which substantially eliminates the charge differential between the particles and its surroundings, reducing or eliminating the lens effect of potential gradient around the particle. If their suggestion is correct, a shadowed specimen would give more valid results than an unshadowed one. Regardless, there are no serious objections to using the shadowing technique and the polystyrene latex as rapid initial checks in order to keep the magnification of an electron microscope constant through the entire period of study.

The size distribution of caseinate particles in individual milk confirmed the majority of results from other researchers[1-8], who gave less detailed information about it. No remarkable differences in the distribution of the particle size were observed among ten samples of individual milk. The sample obtained from pooled milk of 18,000 Kg, which could be considered as average sample, gave fairly good agreement with the average of the individual milk. Holstein milk made up the low-protein group and Guernsey milk made up the high-protein group in the present results. It appeared, however, that there is no relationship between the particle size and the breed or protein concentration of milk.

It is concluded, in this study, that the caseinate particles ranging from 41 to 120 mμ in diameter are most numerous. They amounted from 60 to 80 per cent of the observed particles. The following two considerations, however, should be mentioned: 1) only the caseinate particles of a diameter larger than 21 mμ were considered in this study, and 2) the diameter of imaged particles in electron micrographs were measured and discussed. Particles smaller than 20 mμ in diameter are difficult to identify as caseinate particles. Consequently the size distribution presented in this paper will be modified more or less according to the progress of the electron microscope concerning its magnification power and by further improvement of the preparative method of the specimen. The particle size determined from an image on the electron micrograph is probably larger than the one dispersing in milk because caseinate particles adhering to a supporting film are supposed to be elliptical in cross section when calculated from the shadowing angle and length of the shadow. Further investigation of the cross section of a caseinate particle and the flatness of the supporting film are required.

Summary

Two methods, namely the replica method and the spray method, were successfully applied in preparing a specimen for an electron microscopic study of milk. The effects of dispersing media and the pretreatment of sample on the size of caseinate particles were investigated. No remarkable effects were detected on them with the exception of 0.01 M $CaCl_2$, which gave a slightly higher size of caseinate particles than other dispersing media. The determination of the size distribution of caseinate particles in skim milk was made by spraying or atomizing diluted skim milk (1 : 50 with 0.01 M $CaCl_2$) on a collodion-film-covered grid, which previously had polystyrene latex particles (557 mμ in diameter) on it. More than 96 per cent of the caseinate particles in pooled milk ranged from 21 to 160 mμ in diameter and their average diameter was 85.3 mμ. The average size of the caseinate particles in ten samples of individual milk varied from 76.70 to 103.26 mμ in diameter. The caseinate particles, however, ranging from 41 to 120 mμ in diameter are most numerous in all cases, including pooled milk, and amounted to 60 to 80 per cent of the observed particles.

Acknowledgment

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Explanation of Plates

Fig. 2. Electron micrographs of surface replica of caseinate particles and a fat globule. Shadowed with chromium.

1: Caseinate particles in whole milk.

2: Fat globule in whole milk.

3: Caseinate particles in dialyzed sediment (suspension of caseinate particles obtained from skim milk by centrifugation at 9,600×G for 60 min., at 40°C).

Fig. 3. Electron micrographs of a droplet of diluted (1 : 50) skim milk sprayed by nebulizer.

1: Diluted with ultrafiltrate of skim milk.

2: Diluted with distilled water.

3: Diluted with 0.01 M $CaCl_2$.

4: Formaldehyde was added (0.4%) and diluted with distilled water.

Fig. 4. Electron micrographs comparing polystyrene latex particles (557 mμ in diameter) and caseinate particles in a droplet of skim milk.

1: Unshadowed specimen.

2: Specimen shadowed with chromium.

Fig. 5. Electron micrographs of caseinate in diluted (1 : 50) skim milk. Polystyrene latex particles of 557 mμ in diameter were included as scale. Shadowed with chromium. (The center part of the droplet, with exception of No. 4, was enlarged.)

1: Diluted with distilled water.

2: Diluted with 0.01 M $CaCl_2$.

3: Diluted with 0.4% formaldehyde.

4: Formaldehyde was added (0.4%) prior to dilution with distilled water. The surface part of droplet was enlarged.

Fig. 6. Electron micrographs of caseinate particles in dialyzed and diluted skim milk. Polystyrene latex particles of 557 mμ in diameter were included as scale. Shadowed with chromium. (The center part of the droplet was enlarged.)

1: Formaldehyde was added (0.4%), dialyzed against distilled water for 24 hours, and diluted (1 : 40) with distilled water.

2: The same as 1 but dialyzed against 0.4% formaldehyde.

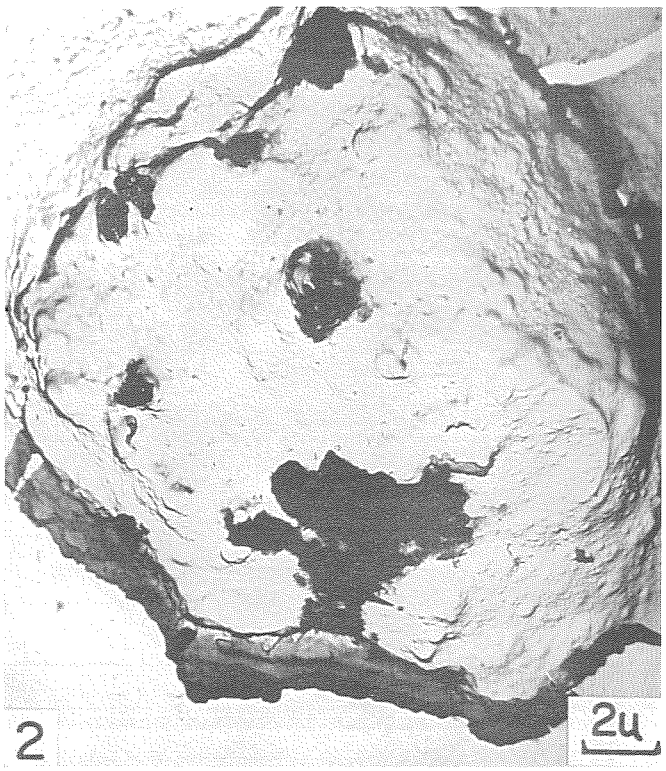
3: The same as 1 but diluted with 0.4% formaldehyde.

Fig. 7. Electron micrograph of stained caseinate particles in a droplet of skim milk diluted 0.2% with phosphotungestic acid.

Fig. 8. Electron micrographs of stained caseinate particles in skim milk.

1: Diluted with 0.2% phosphotungstic acid and placed on a film-covered grid by the single-drop method.

2: The specimen prepared by the single-drop method and treated with the vapor of osmic acid.



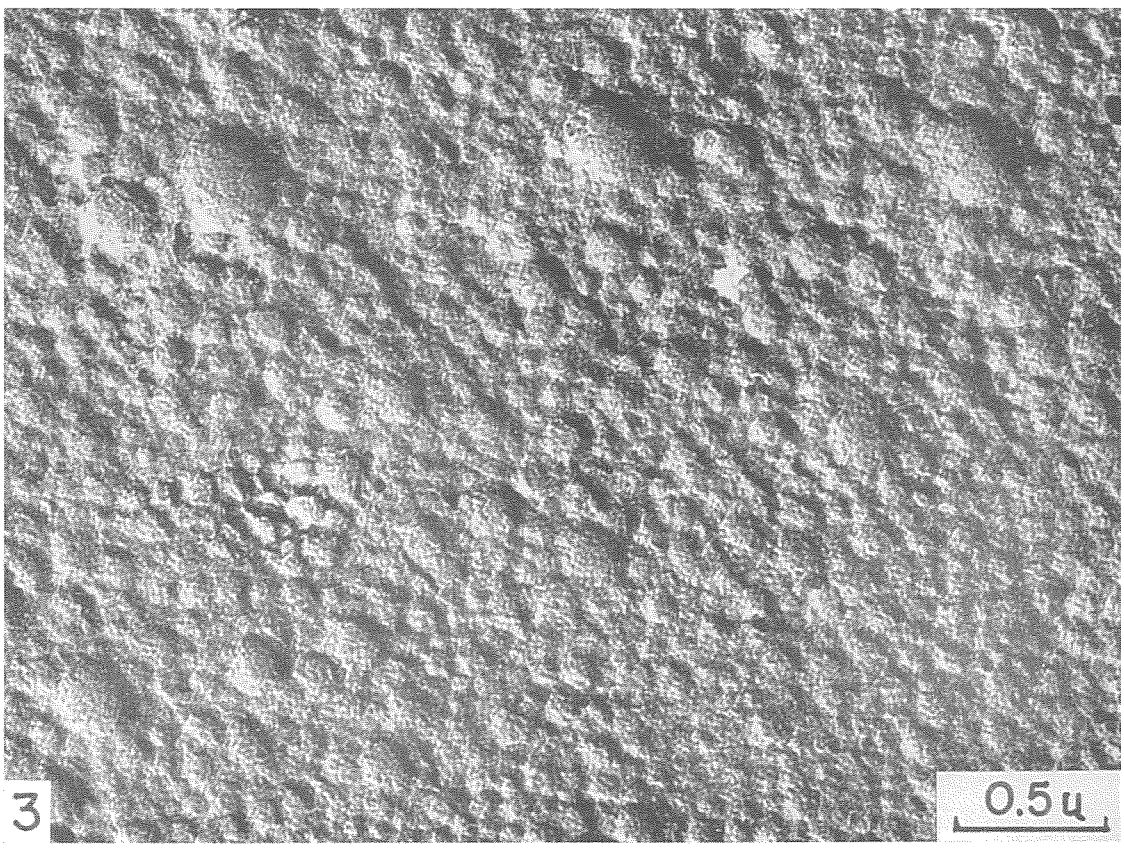


Fig. 2

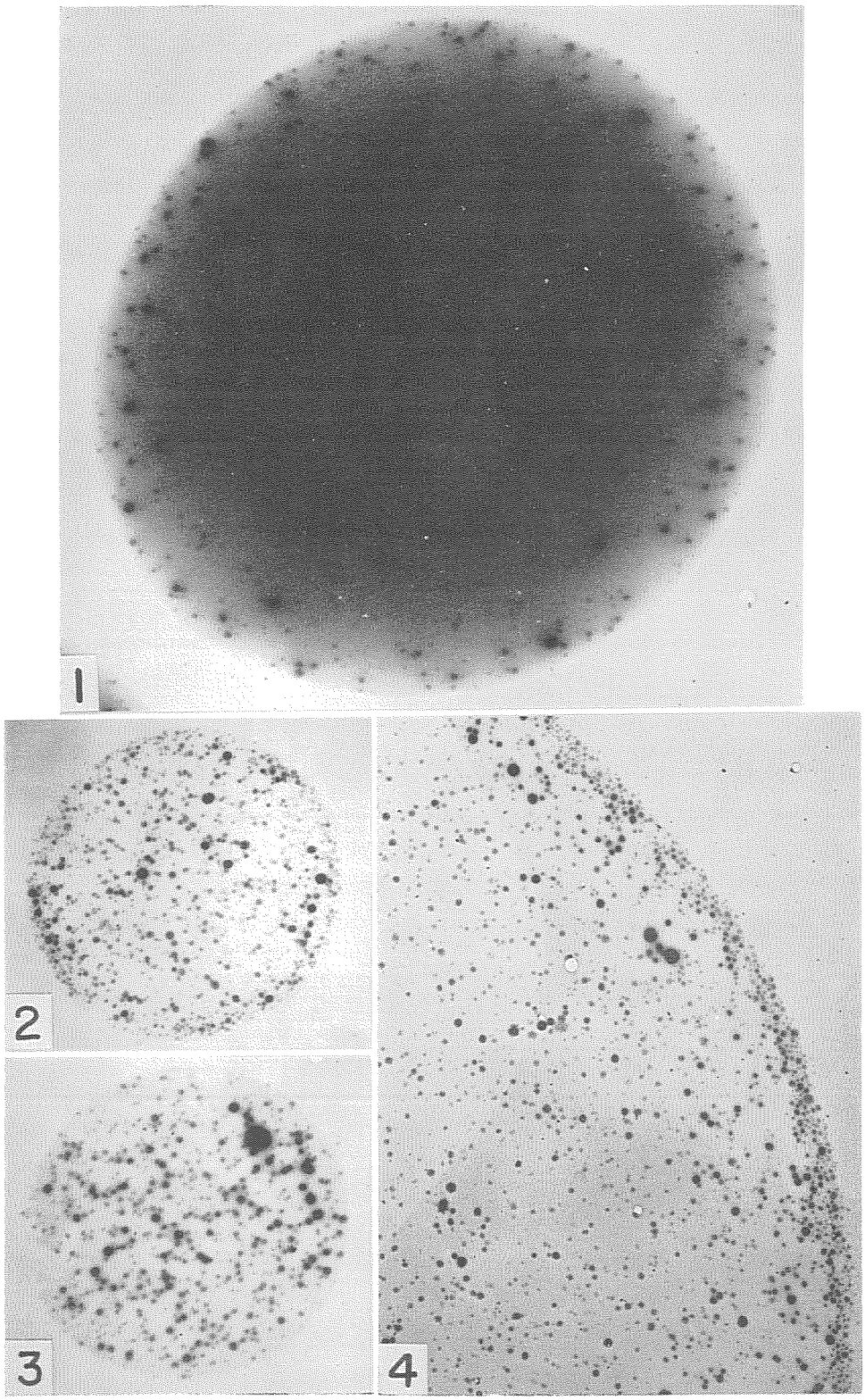


Fig. 3

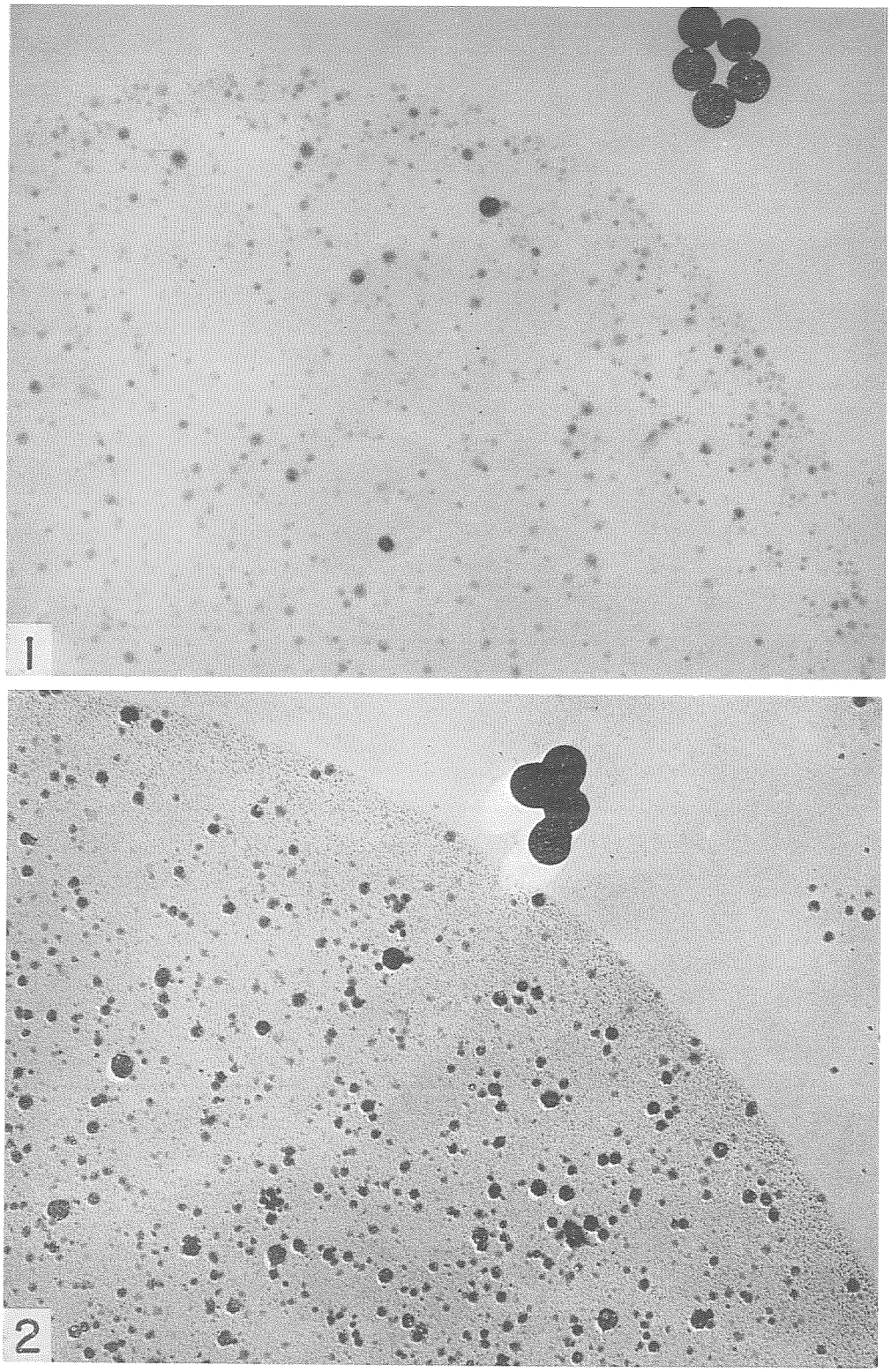


Fig. 4

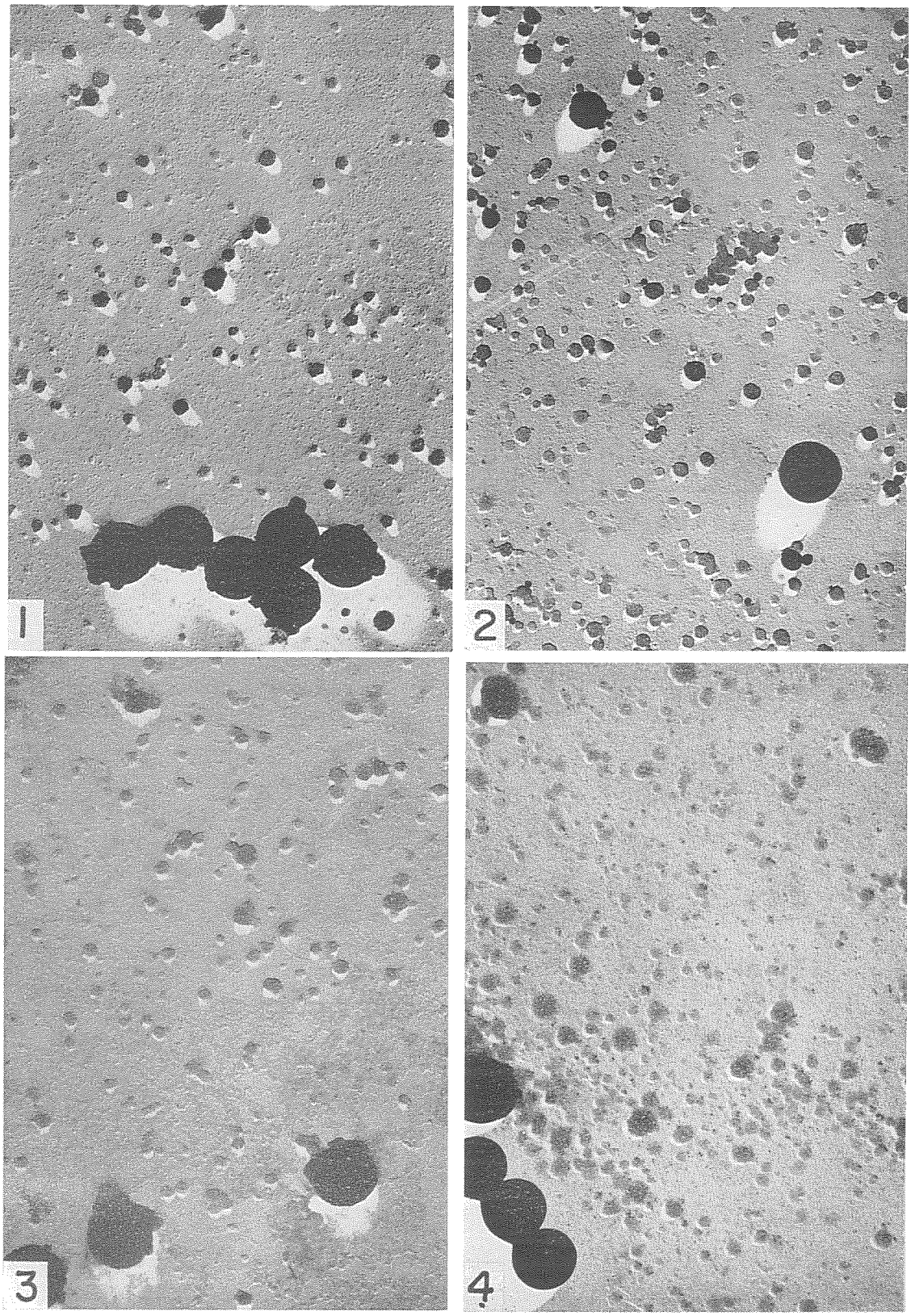


Fig. 5

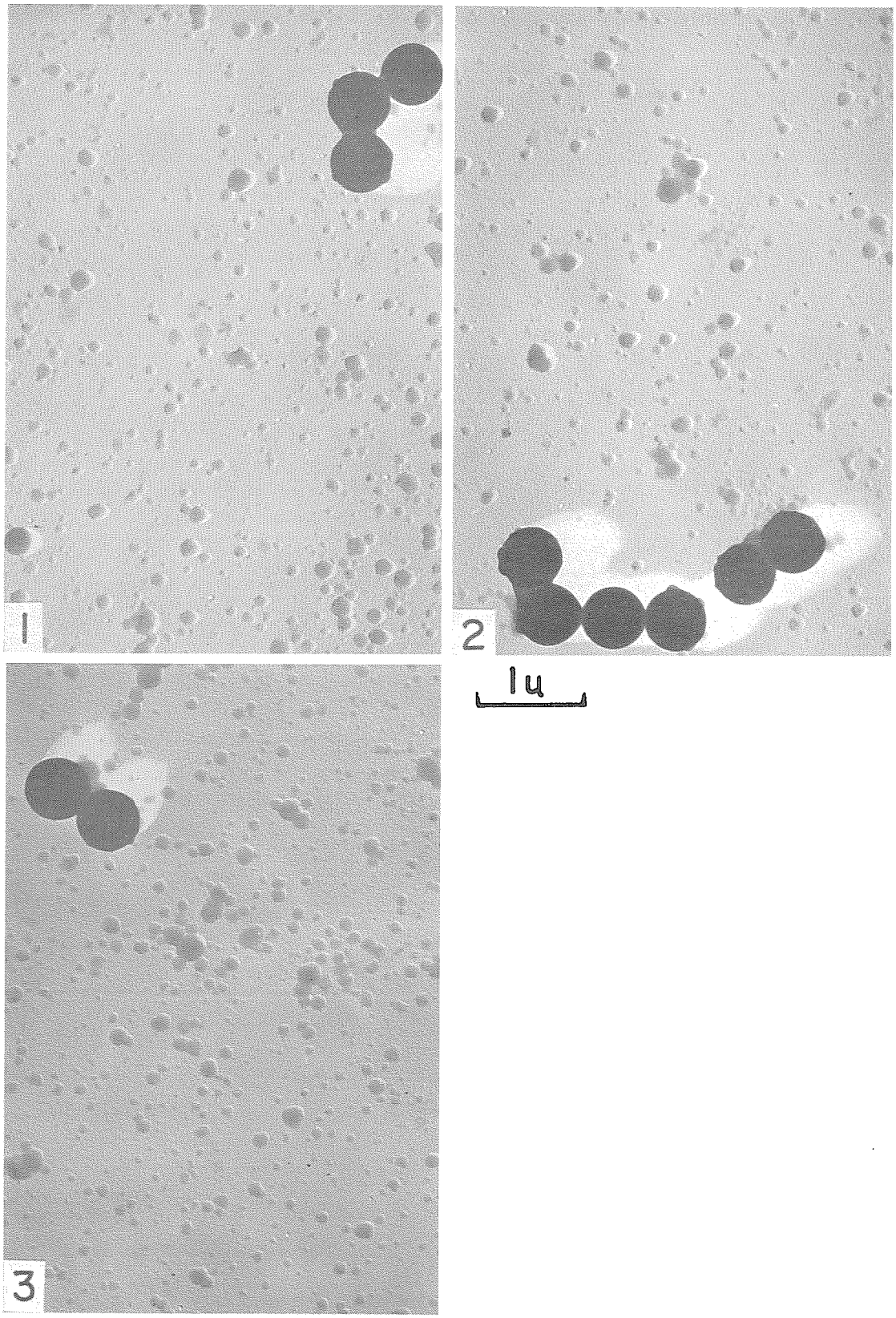


Fig. 6

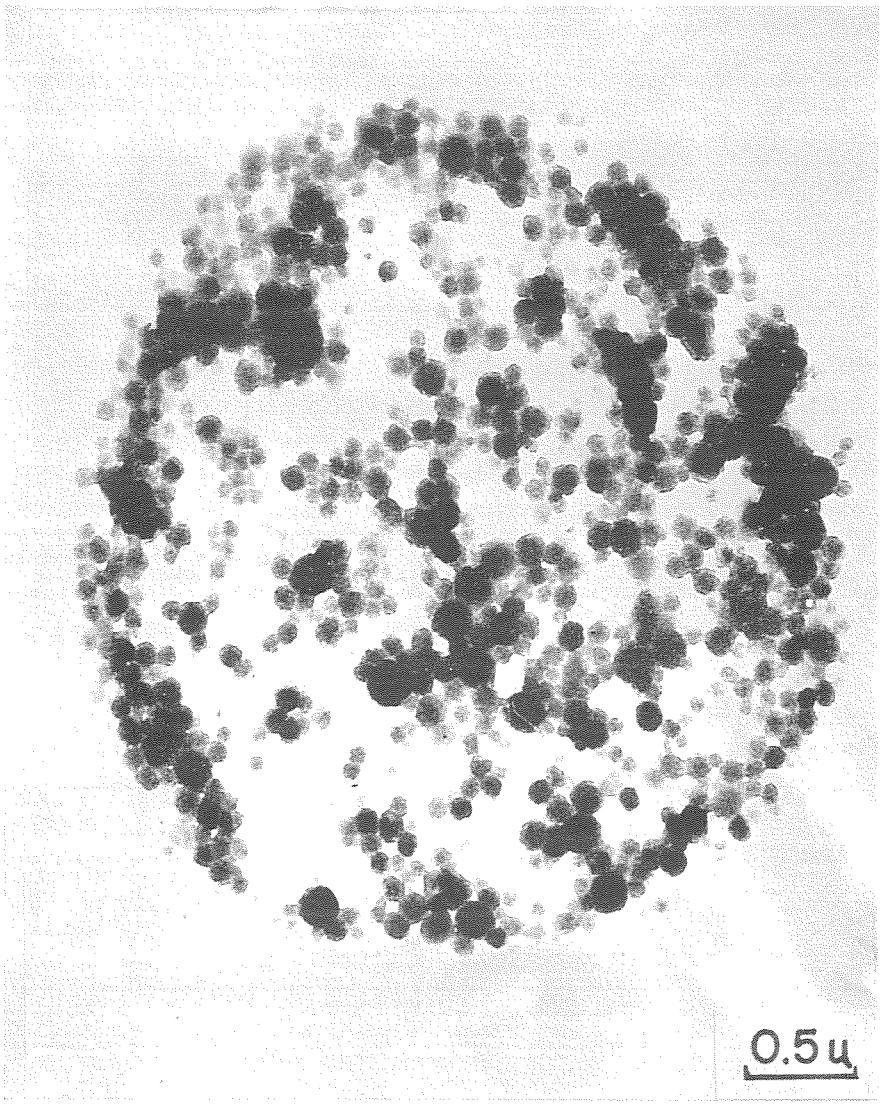


Fig. 7

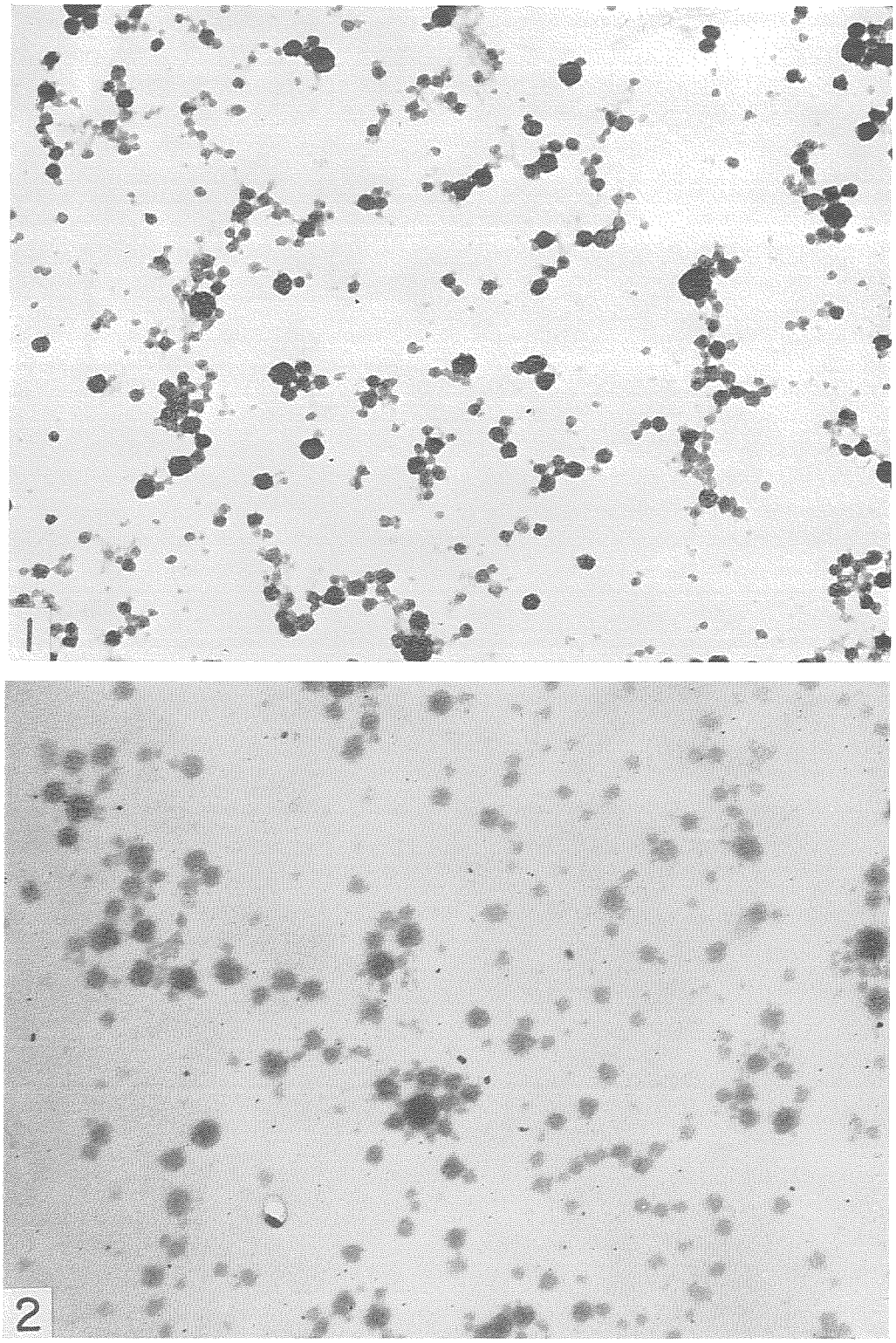


Fig. 8