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Chemistry of Melanin. IV. Electron Micrography of Natural Melanins.*

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Although natural melanins apparently consist of insoluble, heterogeneous aggregates[1,2,3] a water-soluble melanin-containing pseudoglobulin has been separated from pigmented tissue.[4] In this study, we have examined both melanin granules and the melano-pseudoglobulin with the electron microscope to determine (a) whether melanin consists of formed elements or amorphous aggregates, (b) whether structure upon the surface of and within the granule is discernible, and (c) what formal relationship exists between melano-pseudoglobulin and the natural pigment.

Experimental and Results. Electron micrographs were taken at a magnification of 7,600 diameters with a Universal model (R.C.A.) electron microscope. Collateral optical microscopy was carried out with a Zeiss phase contrast microscope† at a magnification of 1085 diameters. Samples of 50 granules were measured to determine size ranges.

Melanin granules from the S-91 transplantable mouse melanoma. Tissue‡ (4-5 g) was ground for 15 minutes with an equal volume of sand and of chilled isotonic saline buffered with 1% of Sorensen's phosphates to pH 6.9. The mixture was diluted to 150 ml with cold saline, then centrifuged at 5° as shown in Table I.

TABLE I.

Time (minutes)	Force (X gravity)	Sediment	Supernatant
3	74	Fraction 1	recentrifuged
15	74	" 2	"
15	159	" 3	"
15	283	" 4	"
15	423	" 5	"
15	636	" 6	"
15	1130	" 7	"
30	1130	" 8	Fraction 9

Under the phase contrast microscope the low speed fractions 2-4 appeared to contain large aggregates and amorphous clumps of both pigmented and non-pigmented materials. In fraction 5 a large majority of the particles were pigmented and in the one μ size range; a small number of aggregates of these granules were also present. In addition, some particles were found which were almost invisible in ordinary light but were demonstrable by phase contrast. The proportion of dark pigmented granules to these "grey" particles was approximately 80/20 Other, highly refractile granules variable in size and shape and ranging from 2 μ to 4 μ were occasionally seen. In fractions 6-9, unit granules comprised the major portion of the particulate material but the proportion of melanin granules to "grey" particles gradually decreased.

Fraction 5 was resuspended in 5 ml of cold distilled water and centrifuged for 15 minutes at 74x gravity. The supernatant fluid was then centrifuged for 15 minutes at 423x gravity. The sediment so obtained, resuspended in cold distilled water, proved to be the most homogeneous preparation of melanin granules obtained from the S-91 melanoma. Roughly 80% of all the elements present were shown by phase contrast to be pigmented. Under the electron microscope this fraction was found to consist principally of elliptical particles, the major axes of which ranged from 0.30 μ to 0.45 μ and the minor axes from

* For the last paper in this series, see Mason, H. S., *J. Biol. Chem.*, Jan., 1948, in press.

1 Smith, D. T., *Bull. Johns Hopkins Hosp.*, 1925, **36**, 185.

2 Herrmann and Boss, M. B., *J. Cell. and Comp. Physiol.*, 1945, **26**, 131.

3 Mason, H. S., *Ann. N. Y. Acad. Sci.*, in press.

4 Greenstein, J. P., Turner, F. C., and Jenrette, W. V., *J. Nat. Cancer Inst.*, 1940, **1**, 377.

† Loaned by the Army Air Forces, Wright Field.

‡ The tumor material was contributed by Dr. Glenn H. Algire.

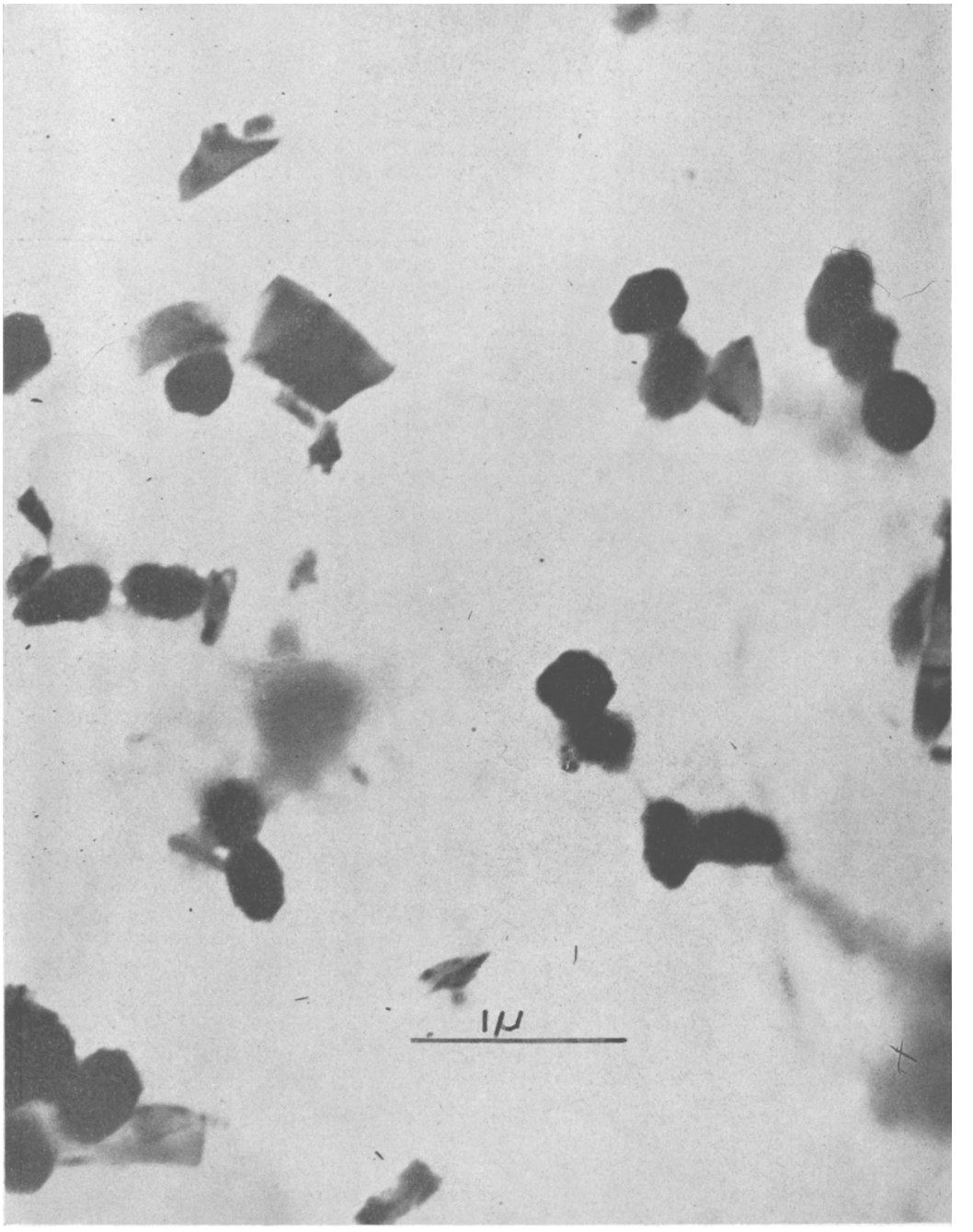

FIG. 1.
Melanin granules from the S-91 melanoma.

0.25 μ to 0.40 μ. The melanin granules themselves appeared almost opaque to the electron beam and no internal structure could be discerned (Fig. 1, 2).

Melanin granules from the Harding-Passey transplantable mouse melanoma. The process utilized above proved effective in concentrating the pigment granules from this source.‡

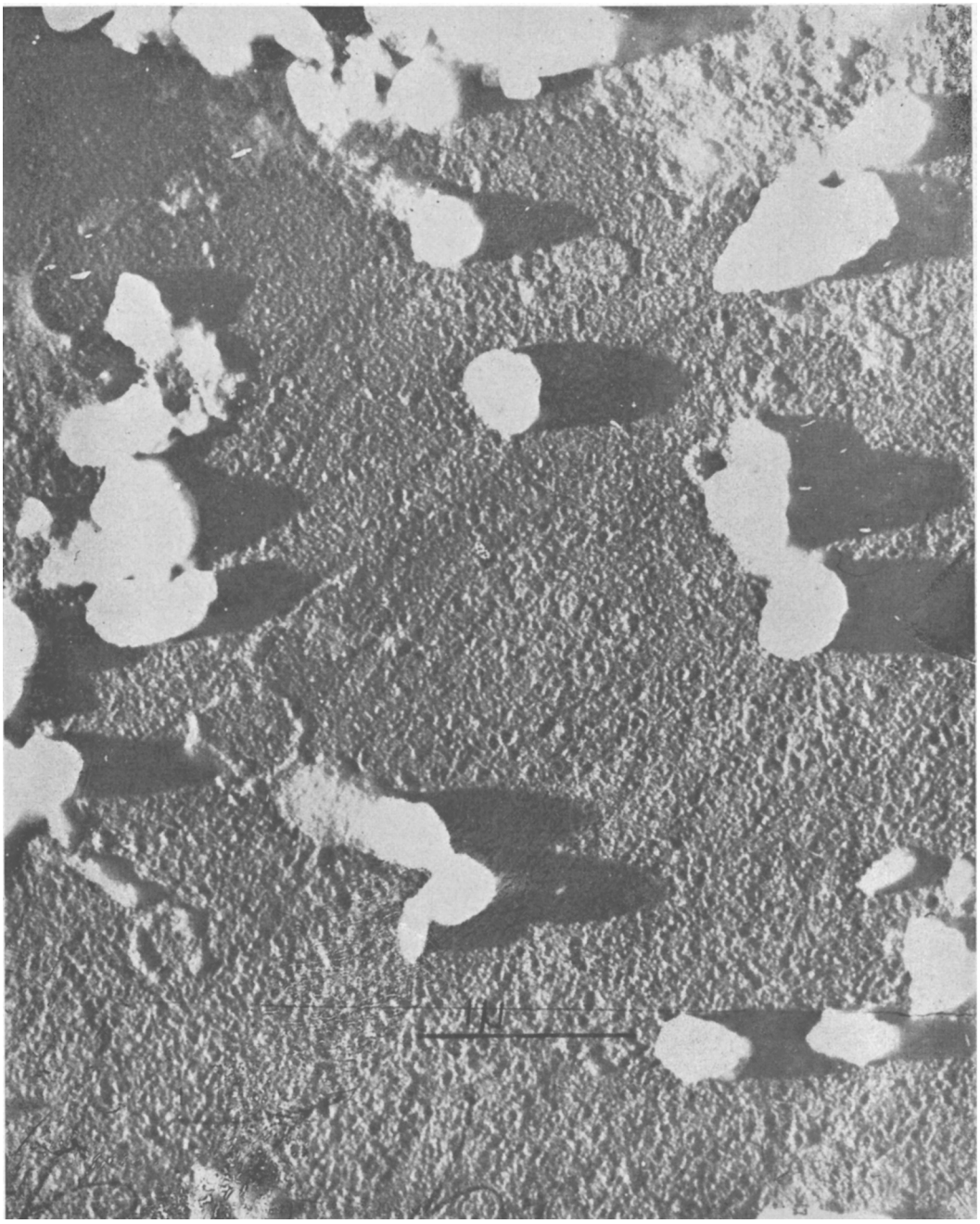

FIG. 2.
Melanin granules from the S-91 melanoma, gold-shadowed at an angle of 1 to 5.

The fractions obtained corresponded in content to those from the S-91 melanoma, and under the electron microscope these melanin granules appeared identical in size range and shape to those from the S-91 melanoma; no internal structure was revealed. (Fig. 3, 4).

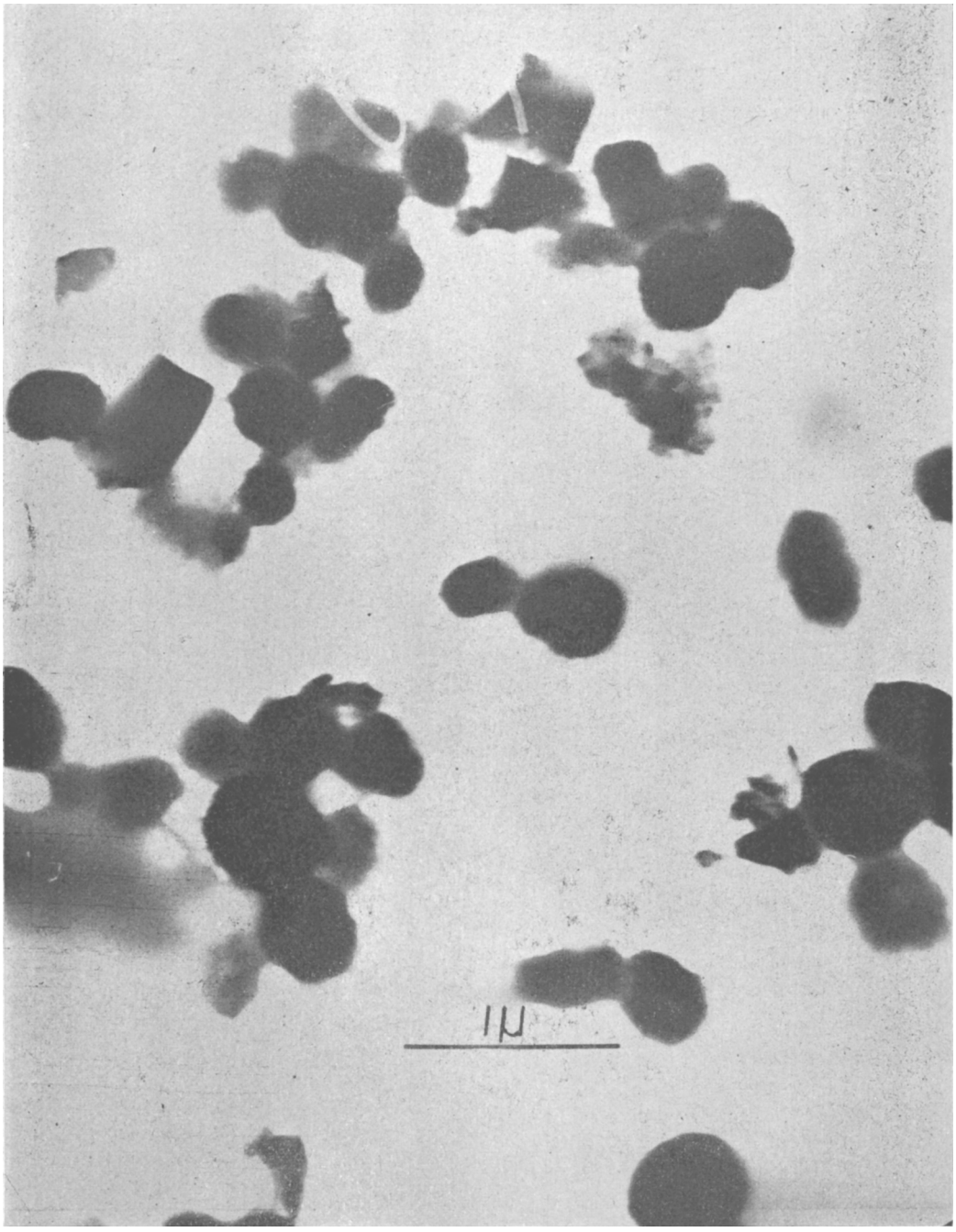

FIG. 3.
Melanin granules from the Harding-Passey melanoma.

Amelanotic melanoma. The specimens[5‡] were prepared and centrifuged in the manner described for the two tissues above. In the phase contrast microscope no evidence of pigmented granules could be found in any frac-

[5] Algire, G. H., *J. Nat. Cancer Inst.*, 1944, **5**, 151.

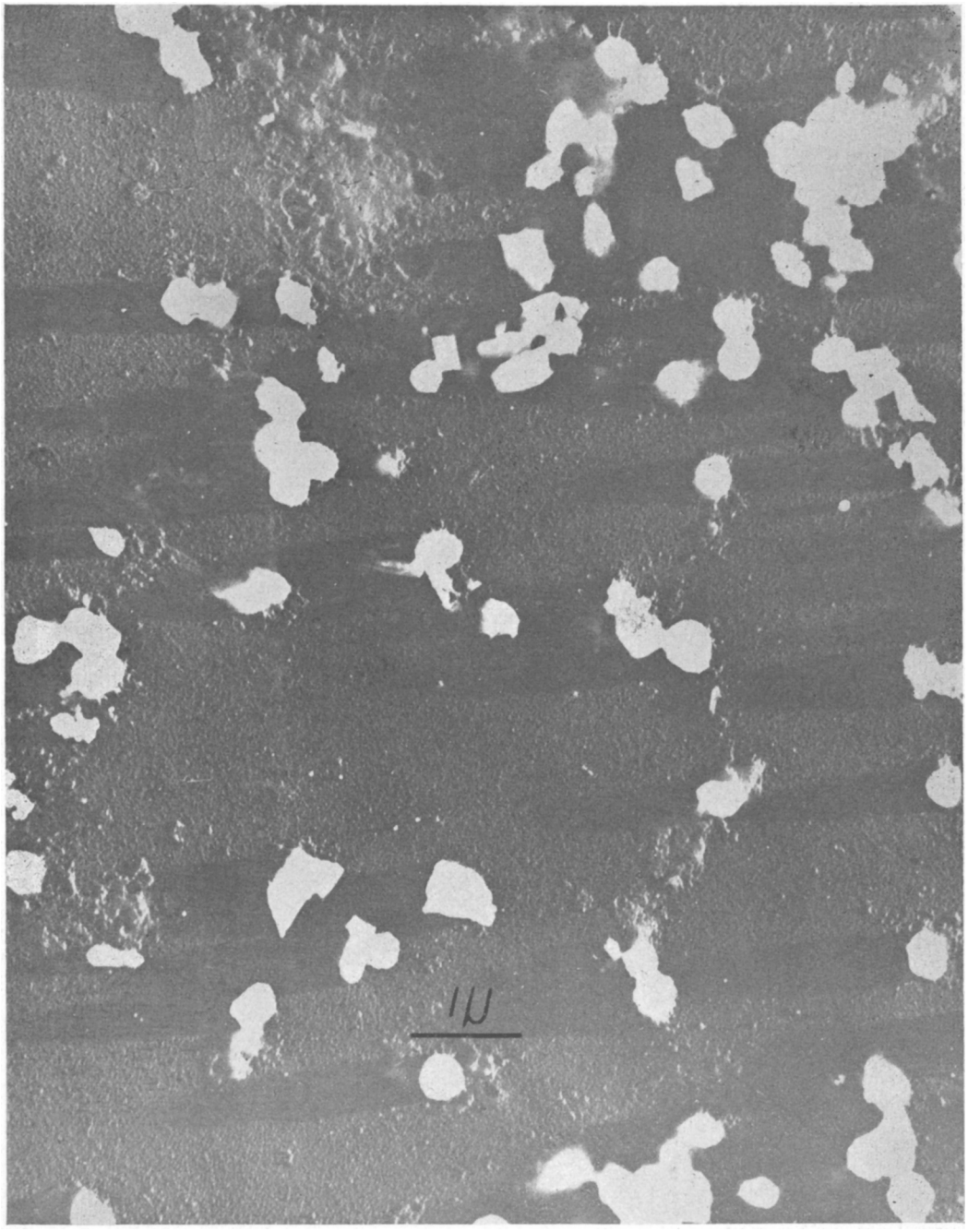

FIG. 4.
Melanin granules from the Harding-Passey melanoma, gold-shadowed at an angle of 1 to 5.

tion but clumped material and unit particles ranging from 0.5 μ to 7 μ were observed. Under the electron microscope, few of these particles corresponded in size or shape to melanin granules from the parent melanoma.

Melanin granules from beef iris, ciliary body

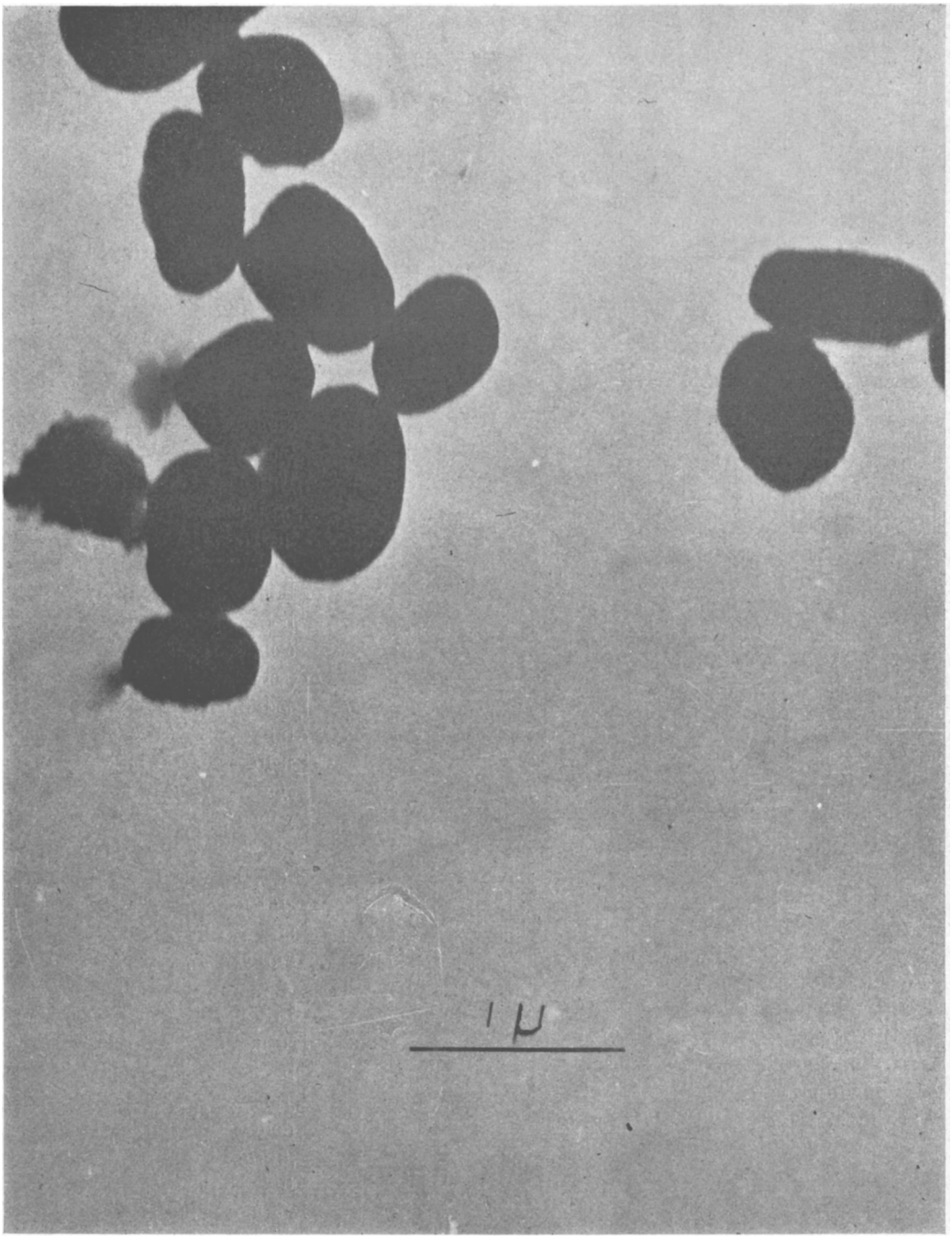

FIG. 5.
Melanin granules from the beef choroid.

and choroid. Ciliary body, iris and choroid were separated from twenty beef eyes.[6§] Each tissue was ground with sand and cold buffered

[6] Friedenwald, J. S., Herrmann, H., and Moses, R., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 421.

§ Beef eyes were obtained from freshly slaughtered animals through the courtesy of the Schluderberg-Kurdle Corp.

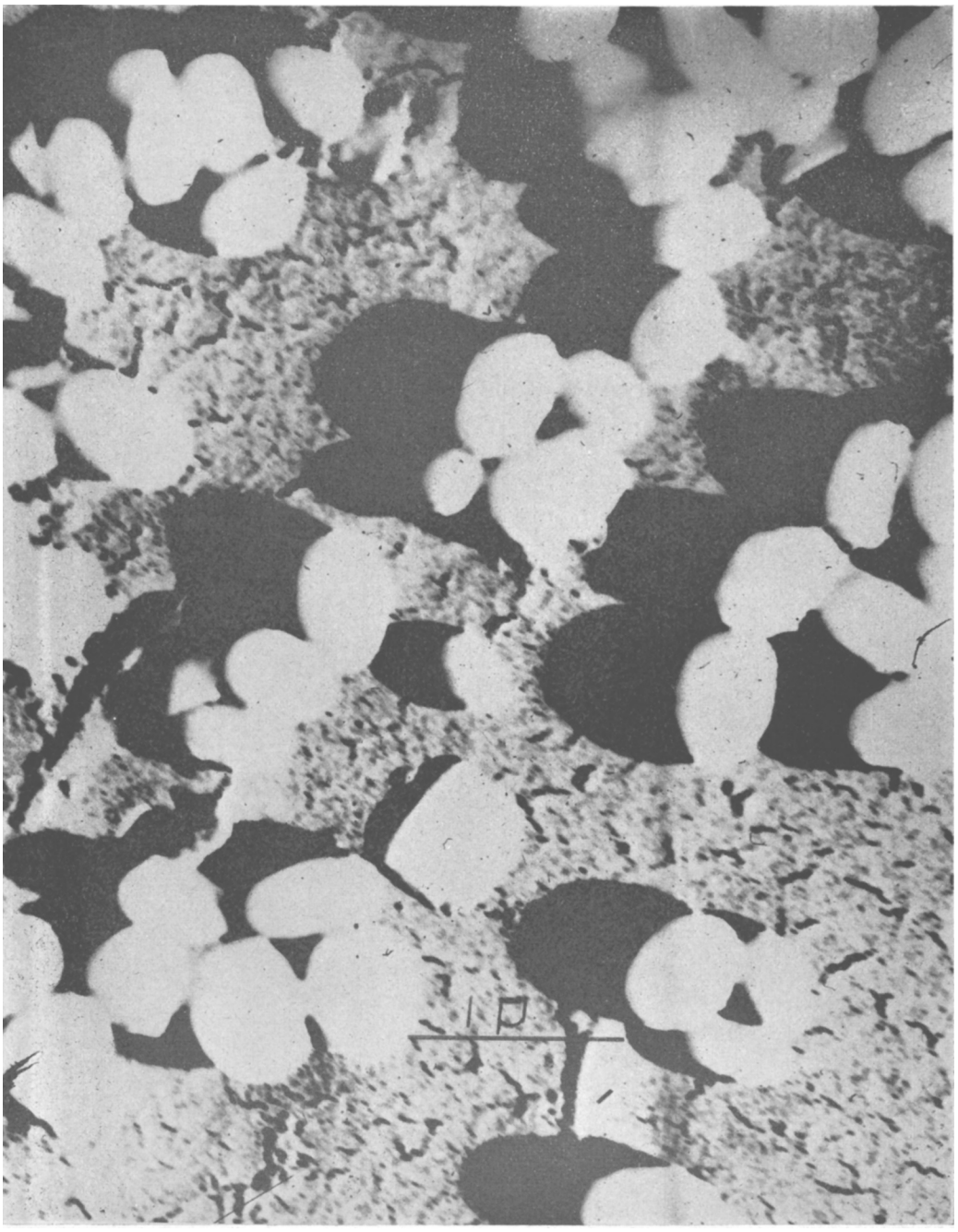

FIG. 6.
Melanin granules from the beef choroid, gold-shadowed at an angle of 1 to 5.

saline as before and the suspensions centrifuged at 74x gravity for 15 minutes; the pellets were resuspended in cold saline and recentrifuged at the same speed. The combined supernatant fluids were spun at 3672x gravity for 30 minutes, and the pellets in each case were resuspended in 10 ml of cold distilled water. The ciliary body preparation then

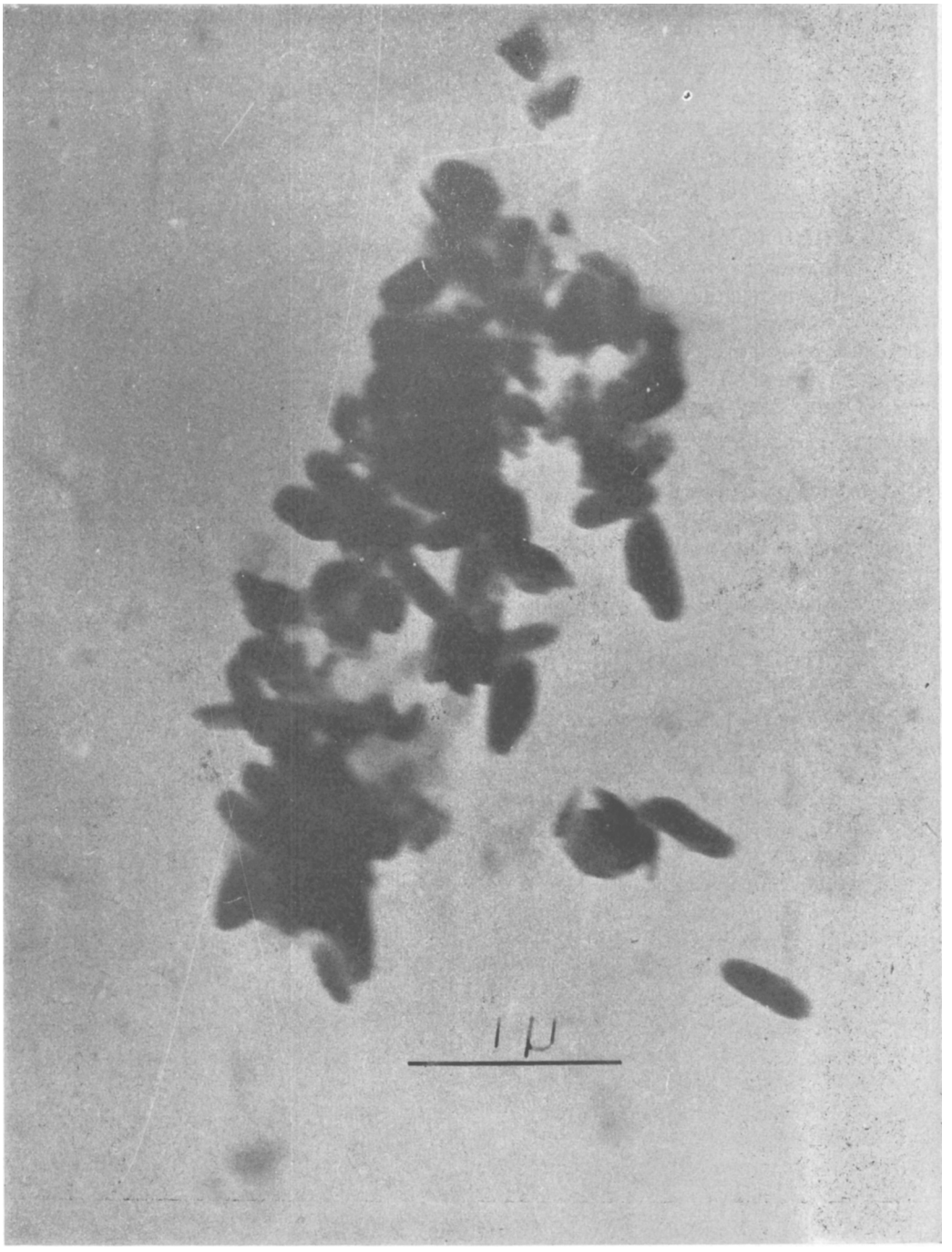

FIG. 7.
Melanin granules from human skin.

corresponded to the granule preparation of Herrmann and Boss.[2] Under the phase contrast microscope it was observed to consist of approximately 95% melanin granules which varied widely in shape and size, together with extraneous material including colorless nuclei.

FIG. 8.
Melanin granules from human skin, gold-shadowed at an angle of 1 to 5.

The particles in each preparation were further sorted by removing aggregated material at 74x gravity (15 minutes), then depositing the major portion of the melanin at 423x gravity (15 minutes) and resuspending the pellet in 5 ml of distilled water. Under the

phase microscope the preparations appeared homogeneous with respect to melanin granules. In the electron microscope the granules varied from circular to elliptical shape, 0.30 μ to 2.00 μ across; they were sharply defined but again failed to reveal internal structure (Fig. 5, 6).

Melanin granules from human skin. A fresh specimen of colored human skin measuring 6 cm by 22 cm was obtained through the courtesy of Drs. John Wirth and Emerson Y. Gledhill. The heavily pigmented epidermis was readily scraped away from the dermis with a blunt scalpel and ground with 2 g of white sand and 5 ml of cold buffered saline for 15 minutes. The resultant slurry was diluted with 25 ml of chilled saline and centrifuged for 15 minutes at 74x gravity. The supernatant fluid was then centrifuged for 15 minutes at 423x gravity. All pigment was deposited in the pellet, which was resuspended in cold distilled water and cleared of aggregated material by centrifuging at 74x gravity for 15 minutes. Under the phase contrast microscope colorless globules about one μ in diameter were observed to constitute about 10% of the bodies visible. Of the pigmented particulates about 35% were rod-shaped and another 35% were small spheres of uniform shape and size. The remainder of the particulate material appeared as large clumps of dark granules. Under the electron microscope globular, rod-shaped, and clumped elements were observable (Fig. 7, 8). The rods varied in size from 0.10 μ x 0.40 μ to 0.18 μ x 0.60 μ. The pigmented globules appeared to have diameters between 0.20 μ and 0.30 μ. The photographic images were uniformly dense and no indication of internal structure could be discerned.

The melanin-containing pseudoglobulin from the S-91 melanoma. This material was prepared by following the directions of Greenstein, Turner, and Jenrette.[4] No difficulty was encountered in repeating the procedure, but the pigmented pseudoglobulin could be deposited almost completely by centrifugation for 15 minutes at 970x gravity. Under the phase contrast microscope the preparation appeared to consist of pigmented particles, much of which was held together in clumps by background material of low refractive index. Nuclei and an occasional whole cell were also seen. Under the electron microscope the pigmented particles proved to be granules of the same size range and shape as those obtained from the S-91 melanoma by the differential centrifugation procedure given above.

Discussion. Melanin granules are characterized with optical microscopes the resolving power of which—approximately 0.2 μ—is of the same order of magnitude as the granules themselves.[7] The electron microscope has a resolving power of the order of 0.005 μ and is, therefore, the instrument of choice for investigation of granule form. However, it does not reveal the presence of pigment as such. We have therefore established the identity of the micrographed particles with melanin granules by optical means. This identity was, in addition, indicated by the relative absence of the typical pigmented forms in granule preparations from the amelanotic melanoma.

The regular and rounded shapes of a representative group of melanin granules now observed with the electron microscope establish these particles as formed elements. The alternative that they consist simply of precipitated aggregates of a metabolic end-product is obviated; melanin is evidently deposited according to a pattern. The demonstration that melanin granules unchanged in shape or size also make up the melano-pseudoglobulin obtained from transplantable mouse melanomas shows that these granules may aggregate in the presence of ammonium sulfate and be redispersed in a measure upon dialytic removal of the salt.

Discontinuities in the micrographic images of melanin granules would give some indication of gross structure. The present series of micrographs however are uniformly dense across the images and conclusions with respect to internal structure cannot be drawn.

Summary. 1. Melanin granules were obtained by differential centrifugation from the S-91 and Harding-Passey transplantable mouse melanomas, from the choroid, ciliary

[7] Russell, E., *Genetics*, 1946, **31**, 327.

body and iris of the beef eye, and from colored human skin. These preparations were characterized with the electron microscope.

2. The melanin-containing pseudoglobulin from the S-91 melanoma was shown to consist of melanin granules unchanged in shape or size with respect to granules obtained by centrifugation.

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Evaluation of a New Test for the Effect of Vitamin P on Capillaries.*

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In our work with rutin[1] we felt the need for a quick test for vitamin P like effects on the capillaries. The method described by Majovski *et al.*[2] appeared to have the advantage of speed and simplicity necessary for the assay of a series of compounds.

The test proposed by Majovski *et al.*[2] consists of the following procedures: Control and test mice are placed in a small jar which is connected by means of a stop-cock and pressure tubing to a five-gallon bottle. The larger bottle is evacuated by means of a vacuum pump to 45 mm of mercury pressure. Upon opening the stop-cock, the pressure in the smaller jar containing the mice quickly drops to 70 mm of mercury pressure. After a 60-second exposure to this decreased pressure the mice are removed and observations are made on the extent of pulmonary hemorrhage. Control mice exhibited a greater degree of pulmonary hemorrhage than did those "protected" by prior administration of certain citrus peel fractions. Thus, it was assumed that this method affords a means of measuring changes in vascular fragility or permeability as influenced by various agents.

In the report of this method, the authors made mention of control animals which failed to conform to the usual reaction and indicated that there seemed to be some correlation between the "presence or absence of food in the stomach and these peculiarities." This observation was repeated by Kibrick and Goldforb[3] who reported "protection" afforded by starving the animals prior to their use in the test as compared with fed mice. Besides, Kibrick and Goldforb[3] found that a number of preparations of the hesperidin group did not afford protection to the mice against pulmonary hemorrhage. They admit, however, that the drugs used may have had varying and unknown contents of vitamin P activity. We felt that the observations of Kibrick and Goldforb did not obviate the usefulness of this method as a test for vitamin P activity, as feeding and fasting of the mice were factors easily controlled.

A few preliminary trials with two groups of fed mice, one of which had received rutin, showed that the animals which had been fed only exhibited gross pulmonary hemorrhages, while those which had been fed and which also had received prior treatment with rutin, showed no pulmonary hemorrhages. Thus it appeared that control of the feeding-fasting factor retained the method's capacity for testing for vitamin P activity.

We have found, however, in the course of our work, that the method is not specific for

* Aided by a grant from the John D. and Fannie K. Herz Fund. The Department is in part supported by the Michael Reese Research Foundation. We are obliged to the Abbott Co. for a supply of rutin.

1 Raiman, R. J., Later, E. R., and Necheles, H., *Science*, in press.

2 Majovski, G. J., Lesser, A. J., Hanson, H. C., Carne, H. O., and Thienes, C. H., *J. Pharm. and Exp. Therap.*, 1944, **80**, 1.

3 Kibrick, A. C., and Goldforb, A. F., *J. Pharm. and Exp. Therap.*, 1944, **82**, 211.