from Cryptococcus which was serologically active up to a dilution of 1:2,000,000.

Summary. 1. Rabbits were immunized with 9 strains of *Cryptococcus neoformans*. After 12 weekly series of injections, 3 strains produced agglutinin titers of 1:320, 4 strains gave titers ranging from 1:10 to 1:40 and 2 strains failed to produce antibodies.

2. On the basis of reciprocal agglutinin absorptions, 3 serologic types of the organism are described. These have been designated as Types A, B, and C.

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17284. Electron Microscopy Study of Chick Embryo Erythrocytes.

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A few electron micrographs of erythrocytes are found in the literature.[1-7] Erythrocytes with cytoplasm made transparent to electrons apparently by the hypotonic solutions which were used in specimen preparation were discovered accidentally in preparations of isolated liver cell nuclei which were being examined in an electron microscope.

Materials and methods. Nuclei of liver cells from healthy, 16-day-old chick embryos were isolated by the differential centrifugation technic of Dounce[8] as modified by Hoerr,[9] and a portion of the final sediment was resuspended in distilled water for immediate deposition upon electron microscope specimen screens. Three other portions were resuspended for ten minutes in 10% formalin, in 2% osmic acid and in 1% silver nitrate, respectively, and specimens of each were prepared as before. The proportion of reagent volume to specimen volume was about 10:1 and seemed excessive, but smaller proportions of reagent and less time for reagent action gave bad results also. Some of the prepared screens which were formalin-fixed only were stained with Harris hematoxylin for 5 minutes; others with 1% safranin for one minute and still others with 1% methyl green for one minute and were washed with distilled water to remove the excess stain. Wet preparations were examined with the ordinary light microscope for control purposes.

Micrographs were taken employing a biased electron gun and an objective aperture with relatively low plate magnifications between 3,000 and 4,000 times. Crystalline residues, which are recognizable in electron micrographs were often observed, and examples of some are indicated at the arrows in Fig. 4, 5 and 12. No effects of electron bombardment were noted upon the morphology of the cells, except that they shrunk when focussed beams were directed upon them, but none of the fields reproduced here have been subject to focussed beams.

Observations and discussion. Unfixed, unstained erythrocytes are identified in this work by their large size, almost filling the whole 2 inches square field at 3,600 times; by their characteristic oval shape; by the centric position and the diffuse, elliptical outline of the nucleus within them; by the proportion of their cytoplasmic to nuclear area; by the homogeneity of their cytoplasm and

1 Wolpers, C., *Naturwiss.*, 1941, **29**, 416.

2 Jung, F., *Klin. Wochsch.*, 1942, **21**, 917.

3 Rebuck, J. W., and Woods, H. L., *Blood*, 1948, **3**, 175.

4 Rebuck, J. W., Woods, H. L., and Monaghan, E. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 220.

5 Barnes, R. B., Burton, C. J., and Scott, R. C., *J. Appl. Phys.*, 1945, **16**, 730.

6 Jones, W. M., *J. Sci. Instr.*, 1947, **24**, 113.

7 Heinmetz, F., *J. Bact.*, 1948, **55**, 823.

8 Dounce, A. L., *J. Biol. Chem.*, 1943, **147**, 685.

9 Hoerr, N. L., *Biol. Symposia*, 1943, **10**, 185.

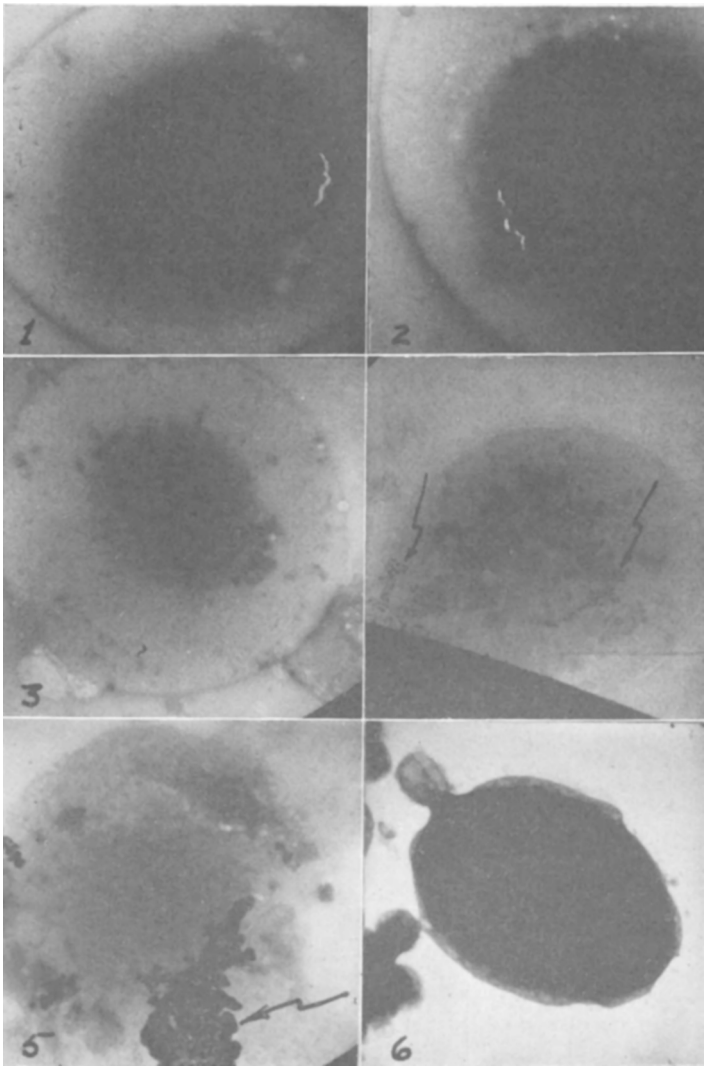

PLATE I.
Electron micrographs of osmosis-hemolyzed chick embryo erythrocytes. × 4000.
Fig. 1, 2, 3, 4 and 5: Unfixed and unstained. Salt residues indicated at arrows.
Fig. 6: Osmic acid fixed where fixation seems to be excessive.

lack of nucleoli in their nuclei; and by the high degree of permeability to electrons of the cytoplasm over the nucleus. They are easily differentiated further from isolated nuclei by their outlines, for where those of the erythrocytes are sharp and well-distinguished, those of the nuclei are diffuse and indistinct.

The nucleus appears clearly in these erythrocytes, except where they have been osmic acid or silver nitrate fixed when detail over almost the whole body of the cell is lost. The nucleus has normal oval appearance in the unfixed, unstained cells and is centric, diffuse-edged and reticular in structure. It shrinks and deforms when it has been formalin-fixed and no structure is seen in it.

Several degrees of hemolysis were apparently provoked by the hypotonic solutions employed in the preparation of the material. Thus, there are different degrees of opacity of the cytoplasm to electrons in each of Fig. 1, 4

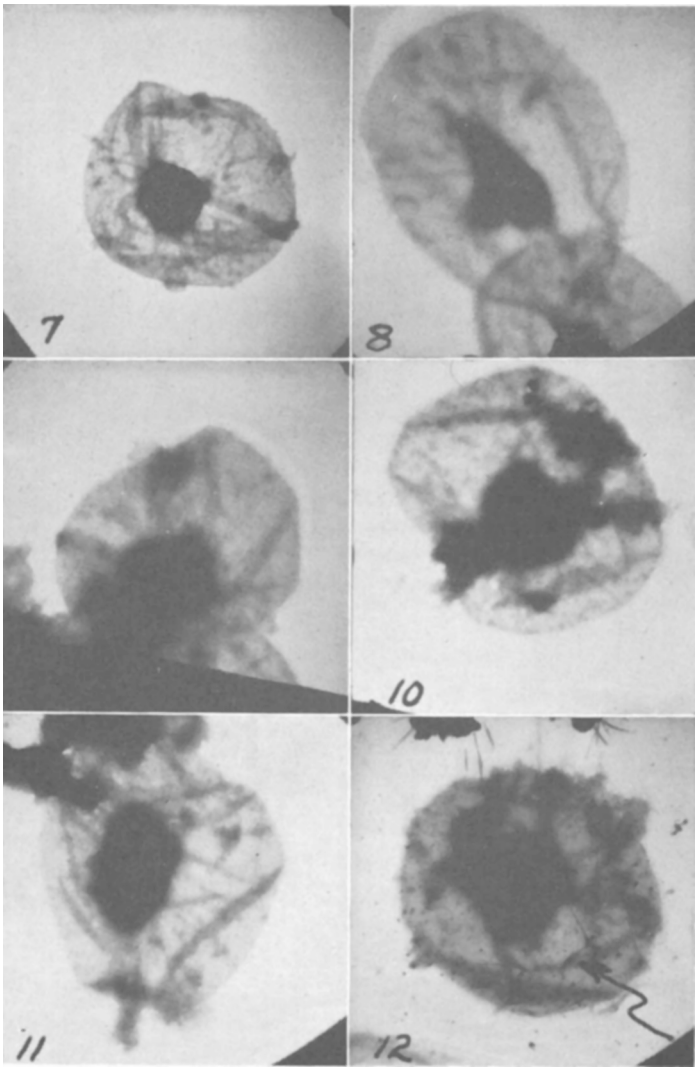

PLATE II.
Electron micrographs of osmosis-hemolyzed, chick embryo erythrocytes, formalin fixed. × 3630.
Fig. 7, 8 and 9: Unstained
Fig. 10: Hematoxylin stained.
Fig. 11: Methyl green stained.
Fig. 12: Safranin stained.

and 5, but the cytoplasm of each of the hemolyzed erythrocytes in Fig. 1 to 5, inclusive, is somewhat transparent to electrons as compared with the known homogeneous opacity of unhemolyzed erythrocytes.[3,4] The sponge-like appearance of the erythrocytes in Fig. 4 and 5 may be the result of an incomplete hemolysis, but it could also support the theory that erythrocytes have a spongy structure. On the other hand, appearance of the images in Fig. 2, 3 and 7 to 12 would support the theory of a balloon structure for erythrocytes.

On several occasions it was possible to rupture the films which supported the erythrocytes and to observe their cross-sections as they were tilted upon them. Under these circumstances the well-hemolyzed erythrocytes

(Fig. 1, 2 and 3) were seen to be extremely flat with outline reminiscent of that of a fried egg. From considerations of electron scattering from such shapes it is very unlikely that the dark outlines in Fig. 1, 2 and 3 can be interpreted as arising from a cell wall or a cell membrane. On the contrary, the most probable explanation for the dark outline and for its non-uniform thickness is that they arise from adsorbed or other material caught about the edge of the cell area as it dries. However, the fact that there is a discrete cell area at all in the micrographs indicates that a cell membrane may exist, although it is invisible in the micrographs. The dark outlines are not visible in the partially hemolyzed or the fixed erythrocytes although the cell areas are again quite discrete. This is evidence, coupled with the previous observations that neither the partially hemolyzed nor the fixed cells flatten out on the films to the same extent that the well-hemolyzed erythrocytes do, and it also suggests again that a cell membrane exists for an erythrocyte, but that it is thin enough physically and atomically to produce negligible electron scattering. Further evidence for the existence of a membrane is given later in the discussion on fixed erythrocytes.

The observations cited so far concerning hemolyzed and partially hemolyzed, unfixed erythrocytes tend to support the important concept of erythrocyte structure, that there is a cell membrane. They also support the following hypotheses concerning the mechanism of erythrocyte hemolysis: (1) that at partial hemolysis an amount of hemoglobin leaves the cell sufficient to allow some electron transmission, so that a spongy appearance becomes manifest over the cell area; at this time (Fig. 4 and 5), no cell membrane is seen, the cell is still distended and a nucleus is dimly visible, and (2) that at the more advanced stages of hemolysis the spongy content contracts upon the nucleus increasing its opacity but leaving a clear area around it, the cell flattens due to lack of cell content and adsorbed material collects about the edge of the cell area as it dries.

In spite of the obvious distortions which are introduced by the fixation (see below) erythrocytes in fixed preparations are recognized and distinguished from the nucleus by use of the same criteria as they are in the unfixed samples. No significant, morphologic differences are detected between erythrocytes fixed with formalin but not stained and those fixed with formalin and stained with hematoxylin, methyl green or safranin. Fig. 10, 11 and 12 show erythrocytes which were fixed and stained with a variety of stains. Fig. 7, 8 and 9 are of a formalin-fixed, unstained sample. Erythrocytes in fixed specimens, both stained and unstained, are obviously distorted due chiefly to the fixation, since unfixed cells are not observed to shrink in the electron microscope at the low intensities at which these were examined. Marked shrinkage of the erythrocytes was also noted in the fixed specimens examined with the light microscope where there was no drying.

Except for the existence of a nucleus, fixed erythrocytes resemble empty bladders in electron micrographs (Fig. 7 to 12), and are similar in this respect to the non-nucleated erythrocytes studied by Wolpers[1] and Jung.[2] The fixed erythrocytes show foldings which are frequently radiated and which indicate that the erythrocytes possess an envelope or membrane. In mammalian erythrocytes at least, this envelope apparently corresponds to the plasma membrane with very little cytoplasm.[10] Such foldings would not be easily seen and indeed are not observed in wet preparations examined with the optical microscope, although they might exist in the wet. It seems logical to explain the occurrence of folds in electron micrographs by assuming that the fixed cell is flabby and its envelope loose. In the act of drying, the envelope falls together on the surface of the specimen film and the folds become visible. In the recently reported work of Chu, Dawson and Elford[11] electron micrographs of fixed chicken red cells are shown which exhibit morphology similar to that reported here.

The reticular structure of the stretched erythrocyte envelope depicted by Wolpers[1]

[10] de Robertis, E. D. P., Nowinski, W. W., and Saez, F. A., General Cytology, Philadelphia, W. B. Saunders Co., 1948, 122.

[11] Chu, C. M., Dawson, I. M., and Elford, W. J., *Lancet*, 1949, CCLVI, 602.

offers a striking similarity to the cytoplasmic structure of some of the hystiocytes described by Rebuck and Woods.[3] Wolpers and Ruska[12] have reported a similar structure for the cytoplasm of the blood platelet hyalomere. One wonders about the relation of fixation and other technical processes to these appearances because in an electron microscope study of tissue culture cells, Porter, Claude and Fullam[13] observed a remarkable variation in the appearance of cytoplasm structure according to the fixation technic employed. Frey-Wyssling[14] points to the possibility that Wolpers' images show artifacts since a similar structure has been observed in the erythrocyte envelope denatured by heat hemolysis.[2]

The outlines of fixed erythrocytes are often observed to be angular. In Fig. 9 the outline is almost hexagonal and approximations to this particular shape were often encountered. The nuclei of fixed erythrocytes are also shrunken and distorted markedly, and are somewhat angular in outline. Nucleus distortions similar to those in the figures of Pl. II were observed in the control wet preparations. This suggests again that the causes of such distortion are to be found not as much in the act of drying as in the action of the fixative on cells which have previously suffered osmotic changes, since the nuclear distortions like the whole-cell distortions are not seen in fresh preparations when the sample is unfixed and osmotic changes have not occurred. The opacity of the nucleus to electrons is increased by the fixation, the contrast vis-a-vis nucleus and cytoplasm being improved thereby. However, this improvement in contrast offers no additional morphological information over unstained specimens; rather, detail is lost due to the increase in opacity of the nucleus. In most of the unfixed as well as the fixed erythrocytes, the nucleus is too opaque to electrons to evidence its structural details, but a coarse reticulum can be observed in some of them in the unfixed samples, Fig. 3 and 5.

A reliable identification of erythrocytes could not be made in specimens which had been treated with either osmic acid or silver nitrate. Both fixatives produced almost homogeneously opaque, oval images in which a reticular structure was evidenced but only at the borders. These erythrocytes had also shrunk and the most marked shrinkage was observed in those treated with osmic acid.

Besides the observations of morphology which have been made in this work and reported in the foregoing discussion, there are a number of points concerning the electron microscopy of biological material[15] which have been illustrated: (a) a biased gun, if used properly and at less than maximum intensities does not affect the subject more than an unbiased one; (b) the primary heating effect of the electron beam is to shrink the cells, but this shrinkage from actual observation is not as drastic, nor does it introduce so many artifacts as does the process of fixation under the experimental conditions; (c) the processes of fixing and staining here tended to complicate the interpretation of electron microscope images rather than to simplify or clarify it; and (d) in general, electron images were likely freer of artifact than light microscopic images when unfixed, unstained samples were used in the former and fixed, stained samples in the latter.

Summary. Electron micrographs of osmosis-hemolyzed erythrocytes from healthy chick embryos are presented. The cells are examined unfixed, unstained; formalin-fixed, unstained; formalin-fixed, hematoxylin stained; formalin-fixed, safranin stained; formalin-fixed, methyl green stained; osmic acid fixed, unstained; and silver nitrate-fixed, unstained. Unfixed, unstained erythrocytes prepared by the technic employed showed a transparent cytoplasm so that the direct visualization of intracytoplasmic parasites might be possible by this procedure. Some nuclear structure was observed, and there is evidence for the existence of a cell membrane. Fixed erythro-

[12] Wolpers, C., and Ruska, H., *Klin. Wochsch.*, 1939, **18**, 1077, 1111.

[13] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

[14] Frey-Wyssling, A., Submicroscopic Morphology of the Protoplasm and its Derivatives, New York, Elsevier Publishing Co., Inc., 1948, 173.

[15] Watson, J. H. L., *J. Appl. Phys.*, 1948, **19**, 713.

cytes clearly showed an envelope. Fixation and drying produced specimen changes, and in the specimens examined the processes of electron microscopy provoked less radical changes than did the fixation.

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17285. Effect of Thymine Desoxyriboside (Thymidine) on Human Pernicious Anemia.

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Shive *et al.*[1] isolated from liver a crystalline substance that inhibited the antagonistic action of methyl-folic acid on the growth of *Leuconostoc mesenteroides* 8293. This substance was identified as thymidine, the desoxyriboside of thymine. Wright *et al.*[2] reported that thymidine could replace vitamin B-12 as a growth factor for certain lactic acid bacteria. Hypoxanthine, adenine or cytosine desoxyribosides[3] and guanine desoxyriboside[4] have also been shown to be able to replace vitamin B-12 as a growth factor for various bacteria.

From this evidence it seemed possible that vitamin B-12 may participate in the synthesis of desoxyribosides, essential to the formation of desoxyribose nucleic acids. Since thymine is effective in combating the hematologic lesions of pernicious anemia, nutritional macrocytic anemia and sprue,[5] the effect of thymidine on patients with pernicious anemia was investigated.

Methods. Three patients with Addisonian pernicious anemia in relapse were treated. All of them had a macrocytic, high color index anemia, with gastric achlorhydria after histamine and megaloblastic hyperplasia of the bone marrow. Reticulocyte counts were done daily, and erythrocyte counts twice a week during the period of observation.

Results. In the first patient, a single injection of 5.3 mg of thymidine was followed by an increase of reticulocytes from 2.0 to 5.0% 4 days later, but by no significant rise in erythrocytes. One week after administration of the thymidine, daily intramuscular injections of 0.001 mg of vitamin B-12 were begun. There was a reticulocyte rise to 10.3% and the red blood cells rose from 2,430,000 to 4,250,000 in the next 13 days.

In the second patient a single injection of 150 mg of thymidine was followed by a rise in reticulocytes from 0.4% to 5.3% on the 4th day, but the red count failed to rise during the week following the injection. Daily intramuscular injections of 0.001 mg of vitamin B-12 were then started. Reticulocytes were 37% on the 7th day and the blood count had risen from 1,600,000 to 4,200,000 on the 21st day of this therapy.

The third patient received a sub-optimal intramuscular dose of 0.00025 mg of vitamin B-12 daily. Reticulocytes increased to 15.5% on the 8th day, and were 1.8% on the 17th day of therapy. At this time, in addition to the vitamin B-12, 5 mg of thymidine was given intramuscularly daily for 9 days. There was a secondary reticulocyte rise from 0.8% on the 18th day to 2.8% on the 21st, 22nd,

* Died May 20, 1949.

1 Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, 1948, **70**, 2299.

2 Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

3 Kitay, E., McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, 1949, **177**, 993.

4 Hoff-Jorgensen, E., *J. Biol. Chem.*, 1949, **178**, 525.

5 Spies, T. D., and Stone, R. E., *Lancet*, 1947, **1**, 174.