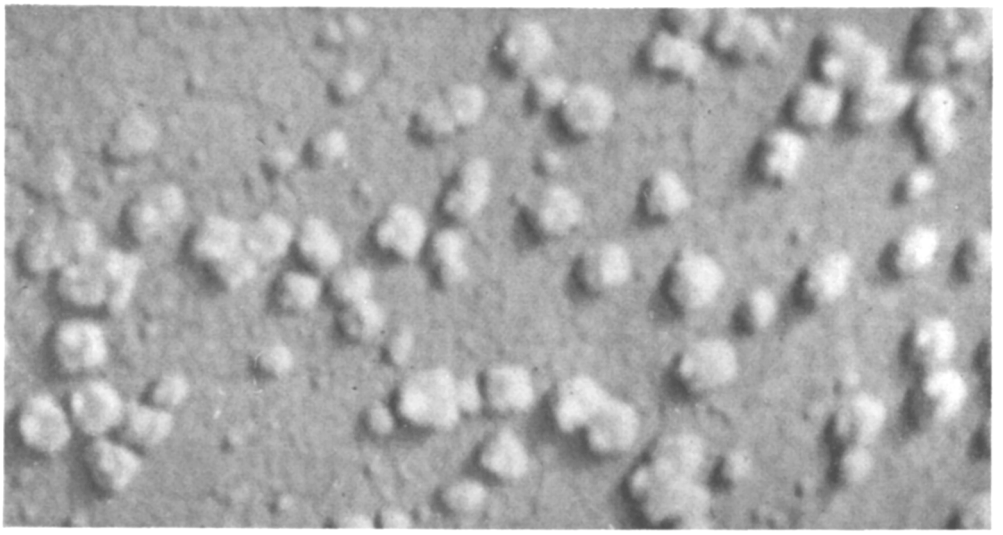

FIG. 1. West Nile Virus shadowed with chromium at arc tangent 2/10. × 150,000.

brains and cords, uniform virus-like particles were demonstrated as shown in Fig. 1. These bodies measure 38-40 mμ by direct measurement and are cube-shaped with irregular contour.

Summary. Studies by electron microscope of brains and spinal cords of mice infected with West Nile virus, B 956 strain, show the virus to be cube-shaped with a diameter of 38-40 mμ. These bodies could not be demonstrated in concentrated normal mice brains and spinal cords subjected to the same procedure of concentration and examination. The bodies from the West Nile preparations resemble the rabbit papilloma virus described by Sharp *et al.*(3). The virus in the concentrated material was infectious for unvaccinated mice but had no effect on mice previously immunized against the West Nile virus.

3. Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 259.

Received October 30, 1951. P.S.E.B.M., 1951, v78.

Composition of Mitochondrial Preparations in Relation to Intracellular Localization of Azoprotein in the Mouse.* (19164)

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(Introduced by J. F. Enders.)
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The tissue localization of labeled proteins has been the subject of extensive investigation, and after parenteral administration, such antigens have been demonstrated repeatedly in the cells of the reticulo-endothelial system and, to a lesser extent, in those of other organs (1-6). It is known that the distribution of

* This investigation was supported in part by a grant from the Division of Research Grants, U. S. Public Health Service, and in part by The Children's Cancer Research Foundation.

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1. Sabin, F. R., *J. Exp. Med.*, 1939, v70, 67.
2. Smetana, H., and Johnson, F. R., *Am. J. Path.*, 1942, v18, 1029.

azoproteins in the tissues of mice and rabbits depends on both the protein and the azo-dye moieties(5,7), and that such proteins are found in the cytoplasm of the involved cells, chiefly in the form of small granules(1-3,5). Recently, Crampton and Haurowitz(8) reported the bulk of injected radio-iodinated antigen to be in the mitochondrial fraction, and suggested that mitochondria are the site of antibody synthesis. In this regard, it seemed of interest to investigate the composition of mitochondrial preparations in an effort to determine whether the small cytoplasmic granules seen after administration of azoproteins are labeled mitochondria.

Materials and methods. Groups of 3 or 4 CF_1 white mice were injected intravenously with 5 to 20 mg of one of the following azoproteins: acriflavine azo-human serum albumin (AHA); acriflavine azo-bovine serum gamma globulin (AGG); and Evans' blue azo-human serum albumin (EBA). (The preparation of these azoproteins is described elsewhere)(9). The animals were sacrificed approximately eighteen hours after intravenous injection of the labeled protein. Microscopic sections showed that the acriflavine azoproteins were localized chiefly in the Kupffer cells of the liver, and the Evans' blue azoprotein in the Kupffer cells and in the cytoplasm of the proximal convoluted tubules of the kidney. The livers of each group were pooled, chilled by being placed in saline solution at 0°C for 10 minutes, and rinsed with chilled normal saline solution and then with chilled 0.88 molar sucrose solution. All subsequent operations were performed at 0°C. The livers were homogenized in a Waring Blendor with 55 ml of 0.88 molar sucrose solution for 45 seconds. The technic for preparation of mitochondrial fractions was essentially that described by Hogeboom and his associates (10). The liver suspensions were centrifuged 3 times at 1,000 G for 15 minutes, and 15 ml aliquots of the final supernatant were centrifuged twice at 20,000 G for 20 minutes in lusteroid tubes. The precipitate in one tube of each group was fixed *in situ,* using a 10% solution of formalin in 0.88 molar sucrose; a duplicate precipitate was resuspended in 0.88 molar sucrose for color determinations. In some instances, a third tube of each lot was centrifuged 5 times at 20,000 G for 20 minutes, and the precipitate fixed *in situ.* Three sets of controls were used: pooled livers of normal mice; pooled livers of mice injected intravenously with 0.025 ml of Higgins' India Ink, and pooled livers of normal mice, to the mitochondrial fraction of which the three azoproteins were added in one-half the amount used for injection. After fixation for 24 hours in the lusteroid tubes, the precipitates were dehydrated and cleared in alcohol and xylol. During this procedure, the tubes were dissolved, leaving the precipitates as thin wafers, which were embedded in paraffin and sectioned. Some sections were examined unstained, whereas others were stained with Altmann's fuchsin, Mallory's phosphotungstic acid hematoxylin, and Regaud's iron hematoxylin methods for mitochondria(11). Sections from various wafers were also stained with McManus' periodate leucofuchsin procedure for carbinols, Gömöri's ferric and ferrous iron procedures, the Prussian blue reaction for sulfhydryl groups, and the methyl-green pyronine procedure for nucleoproteins (12-15).

3. Kruse, H., and McMaster, P. D., *J. Exp. Med.,* 1949, v90, 425.

4. Coons, A. H., Snyder, J. C., Cheever, F. S., and Murray, E. S., *J. Exp. Med.,* 1950, v91, 31.

5. Gitlin, D., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 138.

6. Pressman, D. and Keighley, G., *J. Immunol.,* 1948, v59, 141.

7. Latta, H., Gitlin, D., and Janeway, C. A., *Arch. Path.,* 1951, v51, 260.

8. Crampton, C. F., and Haurowitz, F., *Science,* 1950, v112, 300.

9. Gitlin, D., Latta, H., Batchelor, W. H., and Janeway, C. A., *J. Immunol.,* 1951, v66, 451.

10. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.,* 1948, v172, 619.

11. Lillie, R. D., *Histopathologic technic,* Philadelphia, The Blakiston Co., 1948, p92.

12. Lillie, R. D., *Histopathologic technic.* Philadelphia, The Blakiston Co., 1948, p143.

13. Cowdry, E. V., *Laboratory technique in biology and medicine.* Baltimore, The Williams & Wilkins Co., 1948, p126.

14. Cowdry, E. V., *Laboratory technique in biology and medicine.* Baltimore, The Williams and Wilkins Co., 1948, p233.

TABLE I. Results of Rough Color Comparisons of Azoprotein Content of Various Fractions.

Fraction	% of color of whole liver homogenate			
			Controls with azoprotein added to whole liver homogenate	
	Inj. azoprotein (AHA, AGG, or EBA)	India ink	Washed twice	Washed 5 times
Precipitate after suspension centrifuged 3 times at 1000 G for 15 min	10-20	50-60	5-10	5-10
Mitochondrial fraction	40-50	30-40	5-10	0
First supernatant of mitochondrial fraction	30-40	0-10	80-90	80-90

Results. The results of rough color comparisons of the azoprotein content of the various fractions, using azoprotein solution standards, are indicated in Table I. The distribution parallels that obtained by Crampton and Haurowitz with radio-iodinated proteins in rabbits(8). As can be seen, a relatively large fraction of the injected azoprotein was found in the mitochondrial fraction; however, this was also true of injected India ink. Azoproteins added to the whole liver homogenate were not found in the mitochondrial fraction in significant amounts.

Unstained sections of the wafers prepared from the mitochondrial fractions always showed the azoprotein granules to be concentrated in a narrow zone on the centrifugal edge (Fig. 1), whereas India ink particles formed 2 bands, one on the centrifugal edge and one in the center of the wafer (Fig. 2). Occasionally, the azoprotein layer was discontinuous, reflecting a stellate pattern of dye seen in the whole wafer (Fig. 1, 3 and 4). All 3 mitochondrial stains showed stratification of the material in the wafers; however, the band pattern seen was not the same when the various stains were used on sections of the same wafer, nor was it consistent from one preparation to another. Examples of this are seen in Fig. 3, 4, 5 and 6. In general, the Altmann procedure stained the most granules and the phosphotungstic acid hematoxylin procedure the fewest, whereas the iron hematoxylin procedure gave the best defined granules. In favorable preparations with the 2 hematoxylin stains, the acriflavine-protein colored granules could be seen to form a band which did not take the stain, and with the Altmann stain, Evans' blue-protein colored granules could be seen in a poorly defined zone among the red particles of the fraction. The ferric iron procedure, the only one of the histochemical methods employed which demonstrated stratification in the wafers, did not give bands agreeing with any of the three mitochondrial stains. In some preparations, the McManus procedure stained varying numbers of large irregular structures, presumably small glycogen granules.

Conclusions. These studies substantiate previous reports that a relatively large proportion of injected labeled protein is found in the mitochondrial fraction of homogenized liver(8). Failure to demonstrate in this fraction the azoprotein which was added to the whole liver homogenate indicates that an *in vivo* process is involved and that simple adsorption of the azoprotein by mitochondria does not occur. Results of microscopic study of wafers, made by centrifuging mitochondrial fractions, indicate that the intracytoplasmic granules of azoprotein seen in sections from injected animals(1-3,5) are found predominantly in such mitochondrial fractions. However, the facts that injected India ink particles occur in a similar proportion in such fractions, that the azoprotein granules do not take various mitochondrial stains, and that other impurities can be demonstrated in such preparations, indicate that such mitochondrial fractions do not consist only of mitochondria. This conclusion has also been expressed by previous workers, and such fractions have been subfractionated(16). The differences in re-

15. Cowdry, E. V., *Laboratory technique in biology and medicine.* Baltimore, The Williams & Wilkins Co., 1948, p150.

16. Claude, A., *Ann. N. Y. Acad. Sc.*, 1950, v50, 854.

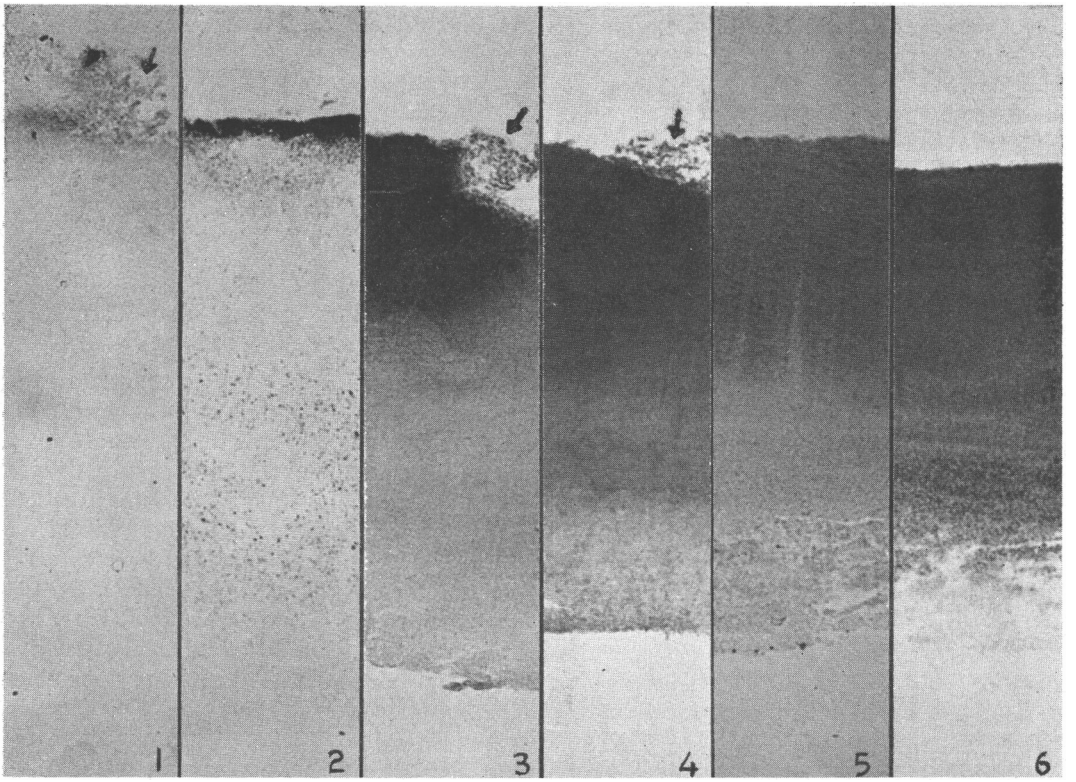

FIG. 1. Unstained section of wafer of sedimented mitochondrial fraction after intravenous injection of acriflavine azo-human serum albumin (AHA), showing concentration of labeled protein on centrifugal (upper) border (× 183). Cleft (arrow) contains loosely packed stained granules, many of which have washed out.

FIG. 2. Unstained section of wafer after injection of India ink, showing bands on centrifugal border and in center of wafer (× 183).

FIG. 3. Same wafer as in Fig. 1, stained with phosphotungstic acid hematoxylin. Note depth of stain along centrifugal border, faint stain of granules in cleft (arrow), and bands in wafer (× 183).

FIG. 4. Same wafer as in Fig. 1 and 3, stained with Altmann's fuchsin stain, showing more extensive stain than with phosphotungstic acid hematoxylin and poor definition of bands (× 183). Granules in cleft (arrow) are fainter than the stained zone.

FIG. 5. Phosphotungstic acid hematoxylin stain of wafer after injection of acriflavine azo-human serum albumin (AHA) and Evans' blue azo-human serum albumin (EBA). A narrow dark line of unstained AHA-labeled granules is seen along the centrifugal border (× 183).

FIG. 6. Same wafer as in Fig. 5, stained with iron hematoxylin. Note dark staining of pale band of Fig. 5 (× 183).

sults with the various mitochondrial stains not only emphasize the inhomogeneity of these preparations, but indicate that such stains do not provide adequate characterization of mitochondria. It is apparent that much more work must be done on the composition of mitochondrial fractions prepared in this way, and on the significance of the results of mitochondrial stains, before cytological and biochemical data on such preparations can be equated with assurance. Since intracytoplasmic azoprotein granules apparently differ in some respects, both physically and histochemically, from other granules in these preparations which fulfill more of the criteria for mitochondria, the occurrence of labeled proteins in mitochondrial fractions does not as yet seem to justify the conclusion that these proteins are attached to mitochondria, or that mitochondria are the site of antibody formation.

Summary. The results of colorimetric and microscopic studies on cell fractions prepared after intravenous administration of various

azoproteins to mice are presented. The azoprotein granules seen intracellularly in the tissues of such animals are present predominantly in the mitochondrial fraction. Evidence that such granules are not labeled mitochondria is presented, and the significance of the variation in the results of different staining technics on mitochondrial fractions is discussed with regard to the homogeneity of these preparations.

Received October 23, 1951. P.S.E.B.M., 1951, v78.

A Semi-Synthetic Medium for Labelling Hemolytic Streptococci with Radioactive Phosphorus.* (19165)

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Although several "synthetic" media for the cultivation of group A hemolytic streptococci, such as those of Bernheimer *et al.*(1), Christensen(2), Stock(3), Dole(4), and Slade and Knox(5), have been reported and appear to be satisfactory for the growth of these bacteria, they are not suitable for studies which require the incorporation of radioactive phosphorus in the bacterial cell. This is due to the fact that their buffer and amino acid nitrogen sources contribute an excessive amount of ordinary phosphorus which would compete with the radioactive form of the element in metabolic processes of the organism.

It is the purpose of the present paper to report the development of a medium which will support excellent growth of streptococci in the presence of a small, but optimal amount of phosphorus in the form of radioactive phosphoric acid.†

Materials and methods. Organisms. The present study was done mainly with group A hemolytic streptococci type 18 which was isolated from the throat of a patient with active rheumatic fever. In the later parts of this study, group A hemolytic streptococcus type 14, which is a mouse-virulent strain (S23/101/4),‡ was also used. The purity of these strains was checked by serological typing at various times during the progress of the experiments. *Inoculum.* A platinum loop, 3 mm in diameter, was used throughout the study. A loopful of an overnight culture of the streptococcus was inoculated into 10 ml of the semi-synthetic medium. The organisms were maintained at first in a serum broth culture. When equivalent growth was finally obtained with the semi-synthetic medium, it was always substituted so as to avoid any possible transfer of traces of substances in the serum broth which might stimulate the bacterial growth. *Criteria of growth.* The appearance of turbidity to the naked eye after cultivation at 37°C for 15 to 18 hours, and the presence of only Gram positive cocci in chains on smear were taken as evidences of growth. Simultaneously with each set of experiments with semi-synthetic media, a tube of serum broth was seeded to serve as a control for the viability of the organisms in the inoculum. *Sterilization of the medium.* After the solid constituents were dissolved, the pH of the solution was adjusted to 7.8; the final volume was made up with distilled water and the

* Aided with a grant from Life Insurance Research Fund.

1. Bernheimer, A. A., Gillman, W., Hottle, G. A., and Pappenheimer, A. M., Jr., *J. Bact.*, 1942, v43, 495.

2. Christensen, L. R., *J. Gen. Physiol.*, 1945, v28, 363.

3. Stock, A. H., *J. Biol. Chem.*, 1942, v142, 777.

4. Dole, V. P., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 122.

5. Slade, H. D., and Knox, G. A., *J. Bact.*, 1950, v60, 301.

† Obtained from the Oak Ridge National Laboratory, A.E.C., Oak Ridge, Tenn.

‡ Kindly given by Dr. Rebecca Lancefield