treated A strain milk and absence of DNA. High-speed centrifugal pellets from defatted and decaseinated strain A milk treated with either ribonuclease or cold 75% ethanol retained tumor-inducing activity. 2) Characteristic particles of 700 Å average diameter, have been observed in sections of high-speed centrifugal pellets of agent-harboring RIII and A strain milk which have shown high tumor-inducing activity. They have also been found following treatment of pellets with fluorocarbon. Similar particles have been observed in considerably smaller number in pellets of Af strain milk without tumor-inducing activity. The relationship of these particles to the origin of mammary tumors of mice requires further study.

1. Dmochowski, L., *Advances in Cancer Research,* Academic Press, N. Y., 1953 v1, 104.
2. ———, *Cancer,* Butterworth & Co., England, 1957, v1, 214.
3. ———, *Proc. IInd International Symposium on Mammary Cancer,* Perugia, 1958, 655.
4. ———, *Acta Union Intern. Contre le Cancer,* 1956, v12, 582.
5. ———, *J. Nat. Cancer Inst.,* 1954, v15, 785.
6. Bernhard, W., Bauer, M., Guerin, M., Oberling, Ch., *Bull du Cancer,* 1955, v42, 163.
7. ———, Guerin, M., Oberling, Ch., *Acta Union Intern. Contre le Cancer,* 1956, v12, 544.
8. Bittner, J. J., *Harvey Lectures,* 1946, v42, 221.
9. Dmochowski, L., Grey, C. E., *Ann. N. Y. Acad. Sci.,* 1957, v68, 559.
10. King, E. J., *Biochem. J.,* 1932, v26, 292.
11. Lowry, O. H., Rosebrough, N. T., Farr, A. L., Randall, R. J., *J. Biol. Chem.,* 1951, v193, 265.
12. Hurlbert, R. B., Schmitz, H., Brumm, A. F., Potter, R. V., *ibid.,* 1954, v209, 23.
13. Burton, K., *Biochem. J.,* 1956, v62, 315.
14. Gierer, A., Schramm, G., *Nature,* 1956, v177, 702.
15. Kirby, K. S., *Biochem. J.,* 1957, v66, 495.
16. Gessler, A. E., *Trans. N. Y., Acad. Sci.,* 1956, v18, 707.
17. Gessler, A. E., Bender, C. E., Parkinson, M. C., *ibid.,* 1956, v18, 701.
18. Epstein, M. A., *Brit. J. Exp. Path.,* 1958, v39, 436.
19. ———, *Brit. J. Cancer,* 1958, v12, 248.
20. Dmochowski, L., Grey, C. E., *Blood,* 1958, v13, 1017.
21. Dmochowski, L., Haagensen, C. D., *Acta Union Intern. Contre le Cancer,* 1955, v11, 646.
22. Bang, F. B., Vellisto, I., Libert, R., *Bull. Johns Hopkins Hosp.,* 1956, v98, 255.
23. Bang, F. B., Andervont, H. B., Vellisto, I., *ibid.,* 1956, v98, 287.
24. Graff, S., Moore, D. H., Stanley, W. M., Randall, H. T., Haagensen, C. D., *Cancer,* 1949, v2, 755.
25. Moore, D. H., Lasfargues, E. Y., Murray, M. R., Haagensen, C. D., Pollard, E. C., *J. Biophys. Biochem. Cytol.,* 1959, v5, 85.

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Use of Contrasting Fluorescent Dye as Counterstain in Fixed Tissue Preparations. (25182)

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One of the major problems in fluorescent antibody studies is the nonspecific uptake of fluorescein dye in formalin fixed tissues which often makes differentiation between specific and nonspecific fluorescence difficult. It was observed that a conjugated bovine antiserum using Lissamine rhodamine RB 200 (LR), according to Chadwick *et al.*(1), not only stained the specific organism but imparted a reddish-orange background to the tissue as well. An investigation was made as to the possibility of using LR-conjugated normal serum as a nonspecific counterstain to eliminate the nonspecific fluorescence of the fluorescein dye.

Materials and methods. Normal bovine, ovine and rabbit whole sera and bovine serum albumin were conjugated with Lissamine rhodamine RB 200* according to Chadwick

* Lissamine rhodamine RB 200 was kindly supplied by Arnold, Hoffman, and Co., Providence, R. I.

et al.(2). For proper conjugation it was necessary to add 0.5% phenol to the serum. One g of LR and 2 g of phosphorus pentachloride (stored over P_2O_5) were ground in a mortar for 5 minutes. Ten ml of acetone (dried over $CaCl_2$) were added and allowed to stand 10 minutes with occasional stirring. The mixture was then filtered. To each 2 ml of diluted serum (equal volumes of serum and 1% phenolized normal saline stored at 4°C for 5 days), 1 ml of carbonate-bicarbonate buffer, pH 9, was added. The mixture was then cooled (0-2°C). With slow stirring, to prevent frothing, 0.1 ml of the acetone filtrate/2 ml of diluted serum was added for 15-20 minutes, care being taken to keep the mixture basic. Stirring was continued overnight at 4°C. The mixture was dialyzed against phosphate buffered saline (pH 7.2) until excess dye was removed. Antisera against bacterial, viral and mycotic agents and goat anti-rabbit were conjugated with fluorescein isothiocyanate according to Marshall *et al.*(3). One volume of LR-conjugated serum was added to 20 volumes of the fluorescein-labeled serum. This was applied to clinical necropsy material from humans and swine as well as material from experimentally infected mice, rats, rabbits and pigeons. The material consisted of impression smears, frozen and formalin fixed paraffin embedded tissue. Also 4 primary and 4 continuous cell culture lines infected with viral or bacterial agents were used. Goat anti-rabbit was applied using the indirect technic. Smears, frozen sections and tissue culture preparations were fixed in 10% neutral formalin for 10-15 minutes, then washed 2 times in buffered saline for 10 minutes. In all preparations, normal controls were used. Stained smears were washed in phosphate-buffered saline, pH 7.2, and mounted in glycerol, pH 7.4. A Zeiss fluorescent microscope with OSRAM HBO 200 light source was used, with either a BG12 exciter and GG4-OG4 barrier filter, or a UG2 exciter and GG4 barrier filter.

Results. The LR was nonspecifically taken up where there was no antigen-antibody reaction. Specific staining by the fluorescein-conjugated antiserum was in no instance prevented or overshadowed in the 14 bacterial, 3 viral, and 4 mycotic systems evaluated. In effect, the specific staining of the fluorescent labeled sera appeared enhanced against a contrasting reddish-orange background. Bacteria in tissues and tissue culture as well as in dried smears took up the LR nonspecifically when not of the homologous species.

Discussion. The problem of nonspecific uptake of fluorescein has plagued many workers. Heretofore it has been necessary to precipitate the globulin fraction and to absorb the conjugated antisera to reduce this phenomenon in tissues and tissue culture, which resulted all too often in lowering the serum titer as well as the volume of the final product. In our experience it was generally unnecessary to precipitate or absorb the sera when using this counterstaining technic. Hughes (4) observed that in the differentiation of neoplastic and non-neoplastic tissue, similar results could be obtained with fluorescein conjugated normal rabbit globulin as with fluorescein labeled protein complexes and that any interpretation of results obtained by fluorescent technics would require caution and careful control staining when evaluating changes attributed to an antigen-antibody reaction. King *et al.*(5) found this to be true. Using normal globulin from 14 species of animals and man, he found results so similar as to be indistinguishable in degree and kind from those observed with rabbit sera. Globulins stained normal tissue but not malignant tissue. Louis(6) confirmed that fluorescein-serum protein may be used as a serologically nonspecific stain for normal tissue and attributed the reaction to presence in the normal cell of a protein complex whose isoelectric point is sufficiently different from that of the serum proteins to allow protein-protein interaction. Therefore as the LR-labeled normal sera react under these circumstances, it is presumed to be a protein-protein physico-chemical interaction, depending on factors other than serological. This system now makes it possible not only to better visualize the sites of antigen deposits but also to detect smaller bacteria and viral aggregates in formalin fixed tissues and tissue cultures.

Summary. A counterstaining method is described that gives a contrasting reddish-orange background when used with fluorescein-labeled antibody systems. It curtails non-

specific fluorescence in tissues and tissue cultures. The possibility of a nonspecific protein-protein reaction is discussed. This reaction apparently plays no part in the serological system to which it has been added.

1. Chadwick, C. S., McEntegart, M. G., Nairn, R. C., *Lancet*, 1958, v1, 412.
2. Chadwick, C. S., McEntegart, M. G., Nairn, R. C., *Immunol.*, 1958, v1, 315.
3. Marshall, J. D., Eveland, W. C., Smith, C. W., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 893.
4. Hughes, P. E., *Cancer Res.*, 1958, v18, 426.
5. King, E. S. J., Hughes, P. E., Louis, C. S., *Brit. J. Cancer*, 1958, v12, 5.
6. Louis, C. J., *idem.*, 1958, v12, 537.

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Characteristics of Growth of Encephalomyocarditis Virus in Cultures of Mouse Muscle.* (25183)

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Encephalomyocarditis virus (EMC) has been cultivated in embryonic mouse tissues by several investigators. Sanders and Jungeblut (1) cultured the Columbia SK strain through 200 consecutive passages in suspended embryonic mouse brain. Both the Columbia SK and MM agents have been propagated in suspensions of embryonic mouse brain, lung, small intestine, cardiac muscle, and tail and in minced chicken embryo by Shean and Schultz(2); no evidence of multiplication was detected when tongue, liver, kidney, spleen or skeletal muscle of embryos were used. Similar observations have been reported by Chambers, Smith, and Evans in tissue cultures of mouse embryo, embryonic intestine, and urinary bladder of hamsters(3). Growth did not occur in mouse intestine or in hamster subcutaneous tissue and skeletal muscle in plasma clot preparations. The encephalomyocarditis virus has also been grown in non-embryonic tissues. Chambers, Smith and Evans(4) recorded its cultivation in suspended human or mouse testicle. Smith and Evans(5) noted that it grew rapidly and produced incomplete degeneration of roller-tube cultures of monkey testicular tissue. Similar findings were reported by Fabiyi(6) using mouse testes as source of cells. Although Mengovirus multiplies in and disrupts Ehrlich ascites tumor cells *in vivo*(7), no evidence of a cytopathogenic effect could be demonstrated *in vitro* by Flanagan and Colter(8). On the other hand, frequent oncolysis of these tumor cells by the encephalomyocarditis virus was observed by Levy and Snellbacker(9). Multiplication of this virus with development of a cytopathic effect in cultures of KB (human epidermoid carcinoma), L, and HeLa cells has been described by Eagle, *et al.*(10), and Jungeblut and Kodza(11): the latter were unable, however, to cultivate the Columbia SK and MM strains on KB cells and the F virus on any but L cells. Our purpose is to report the successful propagation of encephalomyocarditis virus in cultures of abdominal wall muscle of mice and to describe the characteristics of regularly observed cytopathic changes.

Methods. The strain of encephalomyocarditis virus used, was obtained from Dr. Monroe Eaton, Harvard Medical School. It was passed by repeated intraperitoneal injection into mice and a pool of infected brain prepared and stored at –20°C. The muscle for making tissue cultures was obtained from *adult* mice killed by ether. Immediately after death, the skin was reflected and the entire mass of anterior abdominal musculature removed aseptically. After washing twice in Hanks' solution containing 100 units of penicillin and 50 μg of streptomycin, the tissue was minced with sharp scissor until all frag-

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