fluid prior to use in order to remove as much gamma globulin as possible. Evidence for virus multiplication was obtained in only 2 instances. One was a pooled chorion and amnion tissue culture in which virus was demonstrated in the third supernatant (original virus dilution approximately 10^5 times) and the other a spongios tissue culture in the fourth supernatant (original virus dilution approximately 10^7 times). Seven attempts to cultivate the Lansing strain in human foreskin tissue cultures have given negative results, although Weller *et al.*(2) have recently reported positive findings. We feel that our experiments are inconclusive since the foreskins were not received in satisfactory condition and merthiolate was used to disinfect the prepuce prior to circumcision. Additional experiments are planned with more suitable preparations.

Comment. Our findings definitely confirm those of Enders *et al.* on the cultivation of the Lansing strain of poliomyelitis in various human embryonic tissue cultures. We were also able to confirm their claim that this virus is capable of multiplying in cultures composed of non-nervous tissues. The multiplication of the poliomyelitis virus in tissue culture is extremely important because it opens up the possibility of employing tissue culture virus for attempts to produce a poliomyelitis vaccine for human immunization. Undesirable reactions due to inoculation of brain tissue vaccines could thus be eliminated.

Evidence of virus multiplication in placental tissue cultures was obtained in only 2 of 13 experiments. Perhaps the failures were due to the presence of traces of gamma globulin in placental tissues even though these tissues were washed repeatedly with nutrient fluid. The difficulty in removing all antibodies from cells by repeated washings is a common laboratory experience. Moreover, gamma globulin has been shown to have a relatively high neutralizing antibody titer for the Lansing strain of poliomyelitis virus(9,10). Possibly no Lansing neutralizing antibodies were present in the two placenta tissue cultures giving positive results.

Summary. The propagation of the Lansing strain of poliomyelitis virus in various human embryonic tissue cultures was confirmed. The virus was also shown to multiply in cultures composed of non-nervous tissues. Optimum results were obtained with embryonic skin and muscle tissues. The identity of the virus cultivated was established by intracerebral inoculation of mice and rhesus monkeys, histopathologic findings, neutralization tests with Lansing hyperimmune serum, and production of specific active immunity. Evidence of virus multiplication in human placental tissue was obtained in only 2 of 13 tissue cultures. It is suggested that the negative results may have been due to the presence of gamma globulin in the placental tissues.

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Distribution of Azo-Proteins in the Tissues of the Normal Mouse.* (17834)

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The preparation of a highly colored azo-protein, R-salt-azo-benzidine-azo-ovalbumin (1) provided a chemically labelled antigen which could be detected colorimetrically in

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1. Heidelberger, M., Kendall, F. E., and Soo, C. M., *J. Exp. Med.*, 1933, v58, 137.

TABLE I.

Code	Protein	mg/cc	Color
R-OA	R-salt-azo-benzidine-azo-ovalbumin	2	Red
S-OA	Safranin-azo-safranin-azo-ovalbumin	2	Purple
AB-OA	Aniline blue-azo-ovalbumin	2	Blue
R-Gel	R-salt-azo-benzidine-azo-gelatin	4	Red
R-C	R-salt-azo-benzidine-azo-casein	1.7	"
AB-C	Aniline blue-azo-casein	1.7	Blue
AR-C	Alizarin red S-azo-acriflavine-azo-casein	3.5	Purple-red
AC-C	Acriflavine-azo-casein	2	Brown
NR-C	Neutral red-azo-casein	1.5	Brown-red
NRT-C	Neutral red azo-thionine-azo-casein	1.5	Purple

very minute quantities. Several investigations utilizing similarly labelled proteins have since been made to study the distribution of antigenic substances in animal tissues(2-5).

The experiments reported here were undertaken to determine, if possible, the specificity of tissue localization of parenterally administered proteins in normal animals by means of chemical labels which could be detected optically.

Materials and methods. The azo-proteins in Table I were prepared according to the method described(1) for the preparation of R-salt-azo-benzidine-azo-egg albumin.

In preparing the casein group of azo-proteins, 0.17 g of the aromatic amine or diamine in 100 cc water containing 1.5 cc concentrated HCl was diazotized at 5-7°C with 0.09 g $NaNO_2$ and then poured into 125 cc water containing 3.0 g of sodium acetate. 112 cc of the resulting solution was coupled directly to 0.5 g casein in the preparation of AC-C, AB-C, and NR-C, but in the preparation of the other azo caseins, 0.17 g of Neutral Red, 0.23 g Alizarin Red S, or 0.22 g R-salt in 100 cc water was added to the sodium acetate-tetrazonium solution and coupling aided with the addition of 2N Na_2CO_3. 160 cc of the diazonium-azo solution thus prepared was coupled in turn to 0.5 g casein dissolved in 250 cc water at room temperature; coupling was completed with 2N Na_2CO_3.

The R-salt-azo-benzidine-azo-gelatin was purified by acidification with acetic acid and subsequent precipitation with acetone. All azo-proteins were precipitated and redissolved 2 to 4 times and dialyzed against normal saline. These procedures were carried out at 2-5°C. All mice were sacrificed by exsanguination and the tissues were fixed overnight in 12% formalin containing 5% acetic acid. Bouin's fixative or Helly's fluid were used as controls. Gross observations of the distribution of color were made during the procedure of cutting sections of the tissues embedded in paraffin blocks. Sections were counterstained for microscopic study with Mayer's hematoxylin except those containing blue azoprotein and these were left unstained.

Results. Table II gives the gross and microscopic observations made following the injection of groups of mice intravenously with 0.5 cc of several R-salt-azo-benzidine-azo-proteins and disafranin-diazo-ovalbumin. Observation of the presence of azo-protein in the lymph nodes was complicated by the finding that, of several mesenteric and aortic lymph nodes, only one might reveal dye-protein in the fixed macrophages. At the time of these experiments, only a sampling of the mesenteric and aortic nodes was taken from each group of mice. The bone marrow (represented by sections of the femur and sternum) and spleen took on a dark brown color after fixation and, consequently, gross detection of the azo-proteins in these organs was almost impossible.

From the data in Table II, it will be noted that certain differences were observed in the distribution of the various R-salt-azo-pro-

2. Smetana, H., and Johnson, F. R., *Am. J. Path.*, 1942, v18, 1029.

3. Sabin, F. R., *J. Exp. Med.*, 1939, v70, 67.

4. Pratt, H. N., and Gregersen, M. I., *J. Immunol.*, 1941, v40, 163.

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

TABLE II.*

Sites of Localization of Some R-salt azo-proteins and Disafranin-diazo-ovalbumin Following Intravenous Injection into Mice.

No. of mice	Azo-protein	Vol., cc	Time interval	Gross							Microscopic						
				Liver	Kidney	Lungs	Intestine	Heart	Muscle	Nodes	Urine	Liver	Kidney	Lungs	Spleen	Intestine	Heart	Muscle	Nodes	Bone Marrow
3	R-Gel	.5	10 min	0	tr	0	0	0	0	0	+	0	0	0	0	0	0	—	0	—
3	R-C	.5	10 "	0	3	0	0	0	0	0	+	0	x	0	0	0	0	—	0	—
5	R-OA	.5	20 "	2	tr-2	0	0	0	0	2	-	2	x	0	0	0	0	T	4	0
4	"	.5	50 hr	3	tr	0	0	0	0	1	-	4	0	0	1	0	T	T	2	0
3	Dilute R-OA (1:5)	.5	30 min	1-2	0	0	0	0	0	-	±									
3	S-OA	.25	30 "	3	0	1	0	0	-	0	-	2	0	1	0	0	0	—	0	—

(—) Means no observation made.

* The gross observations are recorded arbitrarily from 0 to 4, depending upon the relative intensity of color characterizing the azo-protein; O = no color, 1 = definite but light color, 4 = very intense color, 2 and 3 indicating intermediate grades, tr = trace. Microscopic observations indicate the relative number of cells containing the azo-protein: T = a rare cell, 1 = one macrophage per H.P.F., 2 = one to several macrophages per H.P.F., 3 = almost all the macrophages per H.P.F., 4 = all the macrophages per H.P.F. With respect to the kidney, x indicates observation of color in the tubules; + indicates detection in the urine.

teins though the chemical tag common to them was the same.

In order to answer the question of the importance of the azo-grouping in determining the distribution of the labelled proteins, a series of azo-caseins were prepared and injected intravenously into small groups of mice. The mice were sacrificed an hour after the injections. The results are recorded in Table III.

The possibility of rapid destruction of neutral red-azo-casein, neutral red-azo-thionin-azo-casein and alizarin red-S-azo-acriflavine-azo-casein was then considered, since the failure to detect these azo-proteins in the above distribution study may have been due to this rather than to lack of tissue localization during the time period allowed. Therefore, 6 groups of 2 mice each were injected intraperitoneally with 0.5 cc of the following azo-proteins: AB-C, AC-C, NR-C, NRT-C, AR-C and R-Gel; the mice were sacrificed 28 hours later. The mesentery of each mouse was rinsed with saline, stretched on a glass slide, fixed in formolacetic, stained with Mayer's hematoxylin, and finally mounted in balsam. In each case, the azo-protein was found in large quantities in the macrophages. It became apparent that the macrophages, given a sufficient length of time and suitable conditions, were capable of taking up NR-C, NRT-C, AR-C and R-Gel; failure to detect these azo-proteins in the previous distribution studies, therefore, was not due simply to destruction of the azo-protein.

It will be noted from Table III that differences in the isoelectric points of this series of azo-caseins could hardly be considered sufficient to account for the differences in distribution.

Discussion. The passage of azo-proteins through the mouse kidney was first noticed by Smetana and Johnson(2) and the proteins they used included R-salt-azo-benzidine-azo-casein. These observations seem to be substantiated by the experiments reported here. On the other hand, Pratt and Gregersen(4) reported that they were unable to detect R-salt-azo-benzidine-azo-ovalbumin in the urine of rabbits after intravenous

TABLE III.*

Sites of Localization of Various Azo-caseins and R-salt-azo-gelatin Following Intravenous Injection into Mice. Time interval 1 hour.

No. of mice	Azo-protein	Amt, mg	Approximate isoelectric point, pH	Gross									Microscopic		
				Liver	Kidney	Lungs	Intestine	Heart	Brain	Muscle	Uterus	Nodes	Urine	Liver	Kidney	Lungs
2	AB-C	.85	4.1–4.1	3	tr	0	tr	0	0	tr	tr	3	±	3	0	0
3	AC-C	1.0	4.4–4.2	4	0	0	0	0	0	0	0	tr	0	4	0	0
3	R-C	.85	4.2	1	4	0	tr	0	—	—	—	1	+	1	x	0
4	AR-C	1.75	4.6–4.5	0	0	0	0	0	0	0	0	0	+	0	0	0
3	NB-C	.75	4.5–4.5	tr	0	0	0	0	0	0	0	0	0	0	0	0
3	NRT-C	.75	4.5	0	0	0	0	0	0	0	0	0	0	0	0	0
3	R-Gel	2.0	—	1	1	0	tr	0	—	—	—	tr	+	0	0	0

* Symbols same as for Table II.

administration.

The smallest doses of azo-proteins commensurate with adequate detection were employed to avoid saturation of the cells of one organ which would force into activity the cells of another organ not normally involved. Multiple doses were avoided for the same reason.

In this study, the interval of time between the injection of the azo-protein and the sacrifice of the animal was made as short as possible to reduce to a minimum the possibility of decomposition of the azo-protein *in vivo*. The coupling of a single azo-dye to several different proteins and the observation that differences do occur in the distribution of the azo-proteins thus formed excludes to a great degree the question of decomposition during the time intervals used. If the azo-protein were decomposed and the color group split from the protein molecule, a greater tendency for similarities rather than differences in distribution to occur should have been observed. Heidelberger, *et al.*(6), investigating the production of antibodies in rabbits against R-salt-azo-benzidine-azo-egg albumin, concluded that this dye-protein is not split *in vivo*.

It was observed in these experiments that when several different proteins were coupled to the same dye group, R-salt-azo-benzidine-diazonium, slight differences in the fate of the resulting azo-proteins when parenterally administered to mice were apparent. It was also observed that if a single protein, casein, is coupled to several different azo-compounds, differences in the distribution of the resulting azo-proteins again result. At the time that this paper was in preparation, Hawn and Janeway(7) clearly demonstrated that, when bovine serum albumin and bovine serum γ-globulin were administered intravenously to rabbits, lesions attributable to hypersensitivity were found in differing but characteristic locations for each protein. They concluded that the localization of bovine serum

6. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1934, v59, 519.

7. Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1947, v85, 571.

albumin differed from that of bovine serum γ-globulin. This is in accord with the differences in localization obtained when different proteins are coupled to a particular dye group.

It can easily be argued that the azo-proteins synthesized and used in this study were chemically heterogeneous. The available evidence indicates that this is so(8). Nevertheless, the conclusion that the localization of azo-proteins after parenteral injection may be modified by changes in either the protein or the dye component seems valid.

Summary. The distribution of a series of azo-proteins in mice after parenteral injection was studied by observing the intensity of color in the tissues on gross and microscopic examination. Their distribution appears to be modified either by the azo dye coupled to a particular protein or by the protein to which a particular azo dye is coupled.

8. Gitlin, D., Latta, H., and Janeway, C. A., to be published.

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Development of Reticulum Cells and Lymphocytes in Transplants of Rabbit Lymph Node to Chick Chorioallantois.* (17835)

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The chorioallantoic membrane (CAM) of the developing chick embryo has proven to be a suitable host for various normal and malignant tissues derived from different species(1-4) and it was thought that grafting sections of lymphatic tissue from the rabbit onto the CAM of the developing chick embryo might be feasible. Cervical lymph nodes of guinea pigs were transplanted by Jaffe and Richter(5) into the abdominal wall of rats with regeneration of the lymphatic tissue and with apparent incorporation of the transplanted organ into the economy of the new host.

Technic. The popliteal lymph node of the rabbit was excised and bathed in buffered saline solution containing 300 units of penicillin and 60 μg of streptomycin per ml, then cut into thin slices and incubated in the saline solution at 37° C for ½ to 1 hour. The shell above the natural air sac of 9-day-old embryonated eggs was cut away, and the exposed shell membrane gently teased away from the CAM. A small amount of punctate bleeding of the CAM occurred, which was considered desirable, but eggs which bled profusely were discarded. A slice of lymphatic tissue was placed on the exposed CAM, directly over a surface blood vessel. The egg was then sealed with adequate amounts of Scotch tape and incubated at 37° C in the upright position. After suitable intervals the pieces of lymph node were cut away with the attached CAM and placed in 5% acetic acid Zenker's fixative. Hematoxylin- and eosin-stained paraffin sections were prepared for routine study. Some tissues were also fixed in Serra's solution and stained with methyl-green pyronine.

Results. Embryonated eggs containing slices of rabbit lymphatic tissue were incubated at 37° C for intervals of from 2 to

* This study was supported by a grant from the Commonwealth Fund.

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2. Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1942, v18, 563.

3. Blank, H., Coriell, L. L., and Scott, T. F. McNair, PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 341.

4. Kirber, H. P., Wiener Kirber, M., and Henle, W., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 481.

5. Jaffe, H. L., and Richter, M. N., *J. Exp. Med.*, 1928, v47, 977.