as 0.0103.

Results. Table I lists the results of inorganic phosphate determinations of 13 different samples of dog plasma and 13 different samples of human urine carried out by the proposed method, as compared with duplicate determinations performed simultaneously on the same specimens by the Embden-Fetter strychnine-phosphomolybdate method. Statistical treatment of these values by the application of the t distribution indicated no significant difference between the results obtained.

In the analysis of whole blood, correlation of the results obtained by the two methods on a given sample is good provided that filtration of the precipitate is performed promptly at the conclusion of the one-hour period of standing recommended. However, when this time of standing is prolonged, hydrolysis of organic phosphate appears to take place more extensively in the strychnine-precipitated sample and to make the results from this procedure high as compared with those from the other.

Summary. A method is described for the precipitation of phosphate phosphorus as a barium-polyglycol-phosphomolybdate complex which may be filtered and dried to constant weight at 100°-110°. Evidence is presented to show the applicability of this reaction to phosphate determinations on biological materials, and a comparison is made of this procedure with the strychnine phosphomolybdate (Embden-Fetter) method.

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Effects of Vital Dyes on Early Development of the Amphibian Embryo.

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A vital dye may be used to stain different cells and regions of eggs and embryos. To be successfully employed in this fashion, the dye should be "non-toxic and harmless so as not to destroy or even impair the vital activities and embryonic performance of its carrier" (Weiss[1]). However, it has been shown by Child and his co-workers,[2] in the work on the occurrence of physiological gradients, that so-called "vital" dyes, especially the basic ones, can be quite toxic. Determination of the conditions under which vital staining of eggs and embryos can be successfully accomplished is of interest. The general effects of two basic dyes, Nile Blue Sulfate and Neutral Red, were studied by Detwiler[3] in Amblystoma embryos. Gersch[4] and Gersh and Ries[5] in their work on sea-urchin eggs, found that different concentrations of the acid dye, Trypan Blue, caused severe developmental anomalies. Inhibition of mitosis was shown to occur in the larvæ of *Triton taeniatus* after a one- to two-hour exposure to certain concentrations of Neutral Red (Luther[6]). However, Uschin[7] who studied the same problem in *R. temporaria* tadpoles found no dye effects on mitosis.

In view of the frequency with which the

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2 Child, C. M., *Patterns and Problems of Development*, 1941, The University of Chicago Press.

3 Detwiler, S. R., *Anat. Rec.*, 1917, **13**, 493.

4 Gersch, M., *Arch. Entw. Mech.*, 1937, **136**, 210.

5 Gersch, M., and Ries, E., *Arch. Entw. Mech.*, 1937, **137**, 169.

6 Luther, W., *Klin. Woch.*, 1939, **18**, 682.

7 Uschin, N. P., *Arch. f. Exp. Zellforsch.*, 1931, **11**, 472.

eggs of the leopard frog, *Rana pipiens*, are used in experimental embryology studies, an investigation of their response to exposure to vital dyes under different conditions seemed of practical importance.

Materials and Methods. The dyes used were Nile Blue Sulfate, Neutral Red and Bismark Brown. Fresh stock solutions containing 100 mg of commercial dye† in 1000 cc of solvent (spring water‡) were prepared before each experiment and diluted as required. Eggs of *R. pipiens* (Vermont stock) were obtained in the laboratory by the method of induced ovulation and fertilization described by Rugh[8] and were allowed to develop in finger bowls containing 200 cc of solution; between 20 and 30 eggs per bowl. A total of 2050 eggs were used. Throughout the course of the experiments, the eggs were studied for both character and rate of development. This was recorded in terms of stages of normal development of *R. pipiens* as described by Shumway.[9]

Observations. I. Concentration. Eggs were placed in dye solutions of different concentrations ranging from 1:10,000 to 1:1,000,000 while in the 2-cell stage (st. 3) and were allowed to develop therein up to the complete operculum stage (st. 25). For each dye it was possible to determine a "critical" concentration below which development was completely normal and above which abnormalities and lethal effects began to appear. This critical point varied with each dye, being lowest with Nile Blue Sulfate (1:750,000), considerably higher with Neutral Red (1:250,000) and just slightly higher with Bismark Brown (1:100,000).

Developmental abnormalities produced by exposure to concentrations above the critical levels were of the same nature for each of the dyes. A direct correlation between concentration and time of appearance of the abnormalities seemed to exist; the stronger the concentration the sooner development became abnormal, up to a certain point. However, even with the strongest concentrations used, 1:10,000 (below this point the eggs became so heavily stained that it was impossible to see the cleavage furrows) cleavage was always normal in both character and rate.

The gastrulation stages, beginning with the appearance of the dorsal lip of the blastopore (st. 10) were the first stages in which an effect on development could be noted. In many instances development stopped entirely immediately after the formation of the dorsal lip; in others, development continued but in an abnormal fashion. The effect seemed to be one which involved an interference with the growth of the cells of the animal hemisphere over the large yolk-laden cells of the vegetal hemisphere. Consequently masses of yolk material remained exposed.

All embryos with exposed yolk material which survived beyond gastrulation showed imperfect neurulation. This consisted of an interference with either the formation or the closure of the neural folds. In most cases neural folds could form in the anterior region, and therefore the state of development found in that region was used as a basis for the classification of the embryos into stages. All degrees of abnormalities were found from the cases in which the entire dorsal region of the embryo was occupied by yolk material to those in which only a small yolk plug blocked the closure of the neural folds in the posterior region.

II. Timed exposure to vital dyes. In order to determine, for each concentration, when

TABLE I.

For each concentration is given the length of exposure in hours which did not interfere with normal development. Eggs exposed at stage 3 and maintained at 19 ± 1.5°C. (220 hours represent the time needed to reach st. 25, the end point of these experiments).

Concentration × 1000	Nile blue sulfate	Neutral red	Bismark brown
1:25	—	<3	90
1:50	<3	6	150
1:75	—	12	—
1:100	12	72	175
1:250	24	220	220
1:500	110	220	220
1:750	175	220	220
1:1,000	220	220	220

† Obtained from the National Aniline and Chemical Company, N.Y.

‡ Purchased from the Great Bear Spring Company, N.Y.

8 Rugh, R., *Experimental Embryology, A Manual of Techniques and Procedures*, 1942, New York University Bookstore, New York, N.Y.

9 Shumway, W., *Anat. Rec.*, 1940, **78**, 139.

TABLE II.
Fifteen Hours Exposure to Nile Blue Sulfate.

Hr after fertilization when immersed	Stage No. and description at time of immersion Control and experimental	Description at time of removal. Control and exper.	% showing any abnormality		% surviving through St. 25	
			1:150,000	1:100,000	1:150,000	1:100,000
1	2—grey crescent	mid-cleavage	0	29	97	71
6	6—16 cells	" "	0	25	98	81
22	9—late cleavage	mid-gastrula	48	84	62	33
26½	10—dorsal lip	late "	17	79	78	28
39	11—mid-gastrula	early neural plate	1	80	98	75
45½	12—late gastrula	" " folds	0	76	98	72
63	14—neural folds	neural tube	0	3	100	97

the dye accumulating within the cells reached a concentration incompatible with normal development, eggs immersed at the 2-cell stage, were allowed to remain for graded lengths of time, 3, 6, 12, 18, 24, 36, 48, 67 hours, and then were transferred to fresh spring water for further growth and development. A summary of the maximum exposure to different concentrations which did not interfere with normal development is given in Table I.

A result which might be due to a delayed effect of exposure to the dyes was observed in this series. Eggs exposed for short periods of time to the stronger concentrations developed normally while in the dye solutions and also for some time after removal to fresh medium, but then suddenly became abnormal at the time of gastrulation. For example, eggs which were exposed to 1:50,000 Nile Blue Sulfate from the 2-cell stage to a many-cell stage (a 6-hour exposure) developed normally through this period and for approximately 18 additional hours in fresh spring water. At the end of these 18 hours the eggs reached the dorsal lip stage and the abnormalities already described began to manifest themselves.

III. Effect of stage of development on results of exposure to dyes. Since the first observations indicated a great sensitivity of gastrular stages, a separate study was made of the influence of developmental stage on the results. Eggs were exposed to different concentrations of Nile Blue Sulfate for 15 hours while at different stages of development. In Table II are given the number of hours after fertilization and a description of the development at the time of exposure and removal from the dye solutions.

Of all the concentrations used, 1:100,000 and 1:150,000 were found to be critical for a demonstration of the phase susceptibility (Table II, columns 4, 5, 6, 7). Exposure starting at least one hour before the first cleavage did not delay the onset of division or affect the rate or character of the following divisions. Any departures from the normal occurred at gastrulation, long after removal from the dye. The results were similar for eggs exposed at 6 hours after fertilization (st. 6). Starting at st. 9 an increase in the sensitivity of the eggs was noted as indicated by a rise in the percentage of eggs showing abnormalities as compared with the st. 2 and st. 6 eggs. In a concentration of 1:150,000, the percentage of cases of abnormality decreased in stages 11 and 12. In the higher concentrations of 1:100,000 where it is quite likely that the rate of penetration of dye per unit time was higher, the percentage of abnormality remained large. Nevertheless, the decrease in susceptibility was demonstrable, since the abnormalities produced were less severe and a higher percentage of survival resulted. Of the eggs exposed at st. 11 and st. 12, 75 and 72% respectively were able to survive to st. 25 (the endpoint of the experiment) while of the eggs exposed at st. 9 and st. 10 to the same concentration for the same length of time only 33 and 28% respectively reached the same point. No effect on development was noted when exposure was made at st. 14.

Thus it would seem that in the development of eggs through the neurula stage, late cleavage and dorsal lip stage (st. 9 and st. 10), were the most sensitive to the presence of dye since exposure at these times produced

the largest number of abnormalities and the poorest survival. The question is raised as to whether the high sensitivity seen at st. 9 might not have been due to the fact that in the 15-hour period of exposure, the eggs developed through st. 10 while still in the dye.

IV. Effect of hydrogen ion concentration. The pH of the dye solutions used was found to vary from pH 6.8-6.9 at the time of preparation and to change within a period of 24 hours to pH 7.3-7.4 (probably due to the alkalinity of the glassware used). Therefore, dye solutions buffered to different pHs from pH 4.5 to pH 8.5 were prepared and the staining results compared with those of the unbuffered solutions. It was found that even at high concentrations development proceeded normally through st. 25 in the most acid solutions and that the larvae were only very faintly stained even after long periods. At pH 6.3 to 8.5 the effects were the same as have been described for the unbuffered solutions and also the larvae were all very heavily stained. This agrees substantially with the observations of MacArthur[10] on the staining of *Planaria dorotocephala* with basic dyes and also with the work of Chambers, Irwin and others[11] on the effects of pH on the penetration of dyes into unicellular forms. The phenomenon can be explained either on the basis of the dissociation of the dyes or on the basis of the effect of pH on the particle size (Gordon & Chambers).[12]

Discussion. It has been shown that unless the concentration used, the length of exposure, the stage of development of the eggs, and the hydrogen ion concentration are carefully considered, exposure of the eggs of *R. pipiens* to solutions of vital dyes, Nile Blue Sulfate, Neutral Red and Bismark Brown, may result in abnormal development. This is especially true when the early cleavage stages are used because although cleavage itself remains unaffected the stages which follow are severely damaged.

Nile Blue Sulfate produces good staining but must be used in very low concentrations. Neutral Red is considerably less toxic and Bismark Brown is even less so. As far as could be determined here, there is no difference in the affinity of the 3 dyes for the eggs used. All 3 can readily be seen in fixed sections and also in the living state even though the eggs of *R. pipiens* contain a large amount of dark pigment which has a tendency to mask the color somewhat. These facts would seem to indicate the advisability of using Neutral Red and Bismark Brown in experiments which involve the staining of entire eggs and embryos. The best staining results are obtained by long exposures to weak concentrations rather than by short exposures to strong concentrations. Better results are also produced if the eggs are exposed to the dyes without removal of the jelly capsule. This was also noted by Detwiler[3] for the eggs of Amblystoma. The color produced is uniform throughout the eggs, and the ectoderm is perfectly normal.

Summary. 1. Eggs of *Rana pipiens* were exposed to solutions of the vital dyes Nile Blue Sulfate, Neutral Red and Bismark Brown under different conditions. Concentrations less than the following allowed normal development of eggs and embryos: Nile Blue Sulfate 1:750,000; Neutral Red 1:250,000; Bismark Brown 1:100,000.

2. Developmental abnormalities appeared with use of stronger concentrations than listed above.

3. Early cleavage was unaffected by otherwise toxic concentrations even to 1:10,000. Gastrulation proved to be the critical point in development when toxic effects of the dyes began to produce abnormalities.

4. Short exposure to toxic concentrations, even during unaffected cleavage, resulted in abnormalities appearing at subsequent stages of development.

5. Developmental stages 9 and 10 were more susceptible to toxic effects than were stages 2, 6, 11, 12, 14 using Nile Blue Sulfate of 1:100,000 and 1:150,000 concentrations.

10 MacArthur, J. W., *Am. J. Physiol.*, 1921, **57**, 350.

11 See review of Davson, H., and Danielli, J. F., *The Permeability of Natural Membranes*, 1943, Cambridge University Press.

12 Gordon, H. K., and Chambers, R., *J. Cell. Comp. Physiol.*, 1941, **17**, 97.

6. The pH of the medium was shown to influence the toxicity effects.

7. When Nile Blue Sulfate, Neutral Red and Bismark Brown are used as vital dyes, the factors of concentration, length of exposure and stage of development must be considered to avoid induced abnormalities in development, most of which begin at the time of gastrulation.

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A Method for Producing Experimental Venous Thrombosis.*

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In our attempts to study the genesis and functional pathology of intravascular thrombosis, and to determine the *in vivo* action of heparin on preformed clot, it was found necessary to devise a method of experimental clot formation in animals which produced consistent, predictable results. This report deals briefly with such a method successfully employed in rabbits.

Experimental venous thrombosis has hitherto been accomplished by chemical and mechanical means.[1-4] The introduction of an extraneous factor in chemically-induced thrombosis unnecessarily complicates the proper evaluation of the effect of heparin. Accordingly, mechanical methods of inducing thrombosis are preferable. Vein crushing over an intraluminal silk thread,[5] although reported to be 80% successful, did not give us consistently good results. Similarly, we found stretching of the vein to induce intimal damage[6] successful in only 20% of instances.

The elaboration of a thrombus depends on (a) stagnation of blood, (b) injury to the intima, (c) local release of considerable amounts of thrombokinase. A method utilizing these 3 factors is herewith described. Three kilogram adult rabbits are anesthetized with ether and a midline cervical incision is made. The jugular veins on either side are exposed. Both veins are treated alike. A 3-cm segment of vein is dissected free and the most proximal portion securely tied with a silk ligature. A flat, narrow strip of metal, such as a ribbon retractor, is placed under the vein distal to the ligature and acts as an anvil. The vein is then given 15 to 30 sharp taps with the handle of a Mayo scissors. Brisk bleeding will occur which is readily controlled by gauze pressure. Care is taken not to fracture the vein completely across. When bleeding has ceased, usually in about 2 minutes, a palpable and visible thrombus appears. If clotting does not occur, the procedure is again repeated. Clotting invariably is present after the second series of strokes. All animals are reexamined after 48 hours to reaffirm the presence of clots. Examination and study of these clots, in the gross and in microscopic sec-

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6 Rabinovitch, J., and Pines, B., *Surgery*, 1943, **14**, 669.