final explanation for low yields of virus in comparison to large production of virus precursors may be that a major percentage of virus is inactivated or lost in the extraction procedures.

Of special interest in the experiments described above is the enhancement of NDV replication in HeLa cells treated with actinomycin D. Barry *et al.*(5) reported that NDV replicates to normal titers in actinomycin D-treated chorioallantoic membranes whereas Kingsbury(6) and Granoff (personal communication) observed an inhibition of NDV replication in treated chick fibroblasts. These results are in contrast with those reported here but this discrepancy may be accounted for on the basis of differences in either cell type or virus strain. The slight enhancement of NDV replication in actinomycin D-treated HeLa cells, however, indicates that the sequential events in the replication of this virus can take place independently of DNA synthesis. A reasonable explanation for this enhancement may be that cellular RNA normally competes with viral RNA for biosynthetic sites in the cytoplasm. Cessation of cellular RNA synthesis by actinomycin may remove this competition and permit viral RNA to replicate and direct synthesis of viral protein more efficiently.

Summary. Under conditions in which cellular RNA synthesis was inhibited by actinomycin D, incorporation of C^{14}-uridine took place into RNA of NDV-infected cells and was measured by scintillation counting. In autoradiograph experiments employing H^3-uridine, synthesis of NDV-RNA was demonstrated to take place in the cytoplasm of infected cells.

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Bromination of Phthalein Dyes by the Uterus of the Dogfish, *Squalus acanthias.* (28585)

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The natural occurrence of bromine compounds in marine animals such as the shellfish *Murex brandaris* and *M. tranculus*(1), and the coral fan *Gorgonia verrucosa*(2) indicates that enzymatic bromination may be of importance in their biogenesis. Previously we showed that the uterus of the pregnant spiny dogfish, *Squalus acanthias*, converted phenol red into bromphenol blue after intrauterine administration of the dye(3). Bromination of phenol red was also observed by an enzyme system isolated from the mold *Cal-*

dariomyces fumago(4). This report deals with factors affecting the bromination of phenol red and bromination of chlorphenol red, metacresol purple and fluorescein by the dogfish uterus.

Materials and methods. Fresh pregnant spiny dogfish were kept in a wooden enclosure with free access to sea water at about 14°C. Usually the uterine fluid was withdrawn by pipetting through the cloaca and replaced with a saturated solution of the dye in sea water. At the end of the experiment, generally 48 hours later, the uterine fluid was easily collected by pipetting. In other experiments, the uterus was exposed by an abdominal incision and after replacement of uterine fluid with dye solution, the external opening and the wound were closed, care being taken not to hinder uterine blood supply. In these experiments uterine fluid was collected with a syringe after reopening the incision.

For *in vitro* incubation, a freshly excised uterus was rinsed with sea water and cut into slices about 1 cm^2. A mixture of 3 g of sliced uterus, 2 ml of 0.2 μM phenol red, 1 ml of 0.85 μM NaBr (or 1 ml of sea water) and 8 ml of 0.1 M Tris buffer, pH 7.4 was incubated aerobically at 12 $\pm$ 2°C with shaking for 3 hours, at which time bromination of phenol red reached a maximum. A blank prepared without NaBr was similarly incubated. After incubation, 1 ml of the filtered supernatant was diluted with 2 ml of 0.1 M Tris buffer of pH 8.9 for measurement of absorbancies at 557 mμ and 594 mμ the respective wavelength of maximum absorption of phenol red and bromophenol blue at pH 8.9. Since there is little absorption at 594 mμ due to phenol red, the ratio of A_{594} to A_{557} gives a quantitative measurement of the extent of bromination.

In addition to the uterus, other tissues (see below) of the dogfish were incubated with phenol red *in vitro* as follows(5): The biological material (3 ml) was enclosed in a cellulose sac and placed in a flask containing 1 ml of phenol red, 1 ml of sea water and 8 ml of Tris buffer of pH 7.4. The flasks were incubated together with appropriate blanks prepared similarly but with distilled water in place of sea water. The biological materials included plasma from heparinized blood, a homogenate in 3 volumes of sea water of the fetus detached from the yolk sac, yolk diluted with an equal volume of sea water, and uterine fluid. A reference was provided by incubation with chunks of uterine tissue.

TABLE I. R_f* Values and λmax of Bromination Products.

Compound	System† 1	2	3	4	λmax at pH 8.9
Fluorescein‡	.44		.68		491 mμ
Product§	.05		.24		518
Eosin‖	.05		.25		517
Chlorophenol red‡	.78	.71		.83	573
Product§	.70	.80		.79	592
Bromochlorophenol blue‖	.70	.80		.79	590
Metacresol purple‡	.81	.65		.85	578
Product§	.68	.90		.81	615
Bromocresol green‖	.68	.88		.81	616

* For phenol red, see reference(3).
† 1. NaCl, 3%. 2. BuOH : HOAc : H_2O (4:1:1). 3. NH_4OH, 0.58%. 4. Ammonium carbonate buffer saturated with BuOH.
‡ Compound instilled into uterus.
§ Product obtained 48 hr later.
‖ Authentic bromo-compound.

Paper chromatography was performed on Whatman 3 mm paper in systems listed in Table I. In most cases the uterine fluid was directly applied. Occasionally it was necessary to extract the acidified fluid with ether, wash the ether extract with water and reextract the ether with pH 8.9 Tris. The Tris extract was then applied. Authentic samples were applied or extracted in the same manner for reference.

The spectra of the dyes in the visible region were recorded both before and after intrauterine administration and compared with authentic samples.

Results. Evidence previously reported(3) excludes the possibility that bromination of phenol red was caused by any microorganism in the uterus of the dogfish. Under *in vitro* conditions described in Methods only uterine tissue showed activity in brominating phenol red. This activity seemed to reside primarily in the uterine epithelium, being 4 times as active as uterine muscle. The slight activity obtained with uterine muscle might be due to incomplete removal of epithelial tissue dur-

ing the preparation. Uterine epithelial tissue and muscle boiled for 5 to 10 minutes were completely inactive.

For *in vitro* bromination of phenol red by uterine slices, the pH optimum was determined in glycine sulfate, acetate, and Tris buffers of pH 2.8 to 8, all of 0.1 M. The optimum pH is about 7.4 close to the body pH of the fish(6).

The *in vivo* bromination experiments were extended to include several other phthalein dyes, namely, chlorophenol red, metacresol purple, and fluorescein. The Rf values in various systems and the λ max of these dyes in 0.01 M Tris buffer of pH 8.9 both before and after the intrauterine administration are listed in Table I together with those of the authentic bromo-derivatives. A bathochromic shift is associated with bromination in all cases, and positive bromination is readily recognized by the striking change in color.

With the exception of fluorescein, the dyes studied are phenosulfone phthaleins. Since fluorescein was as readily brominated, the sulfonic acid moiety could be replaced by the carboxylic group without adverse effect.

To further elucidate structural requirements of *in vivo* bromination, solutions of m-hydroxybenzoic and p-hydroxybenzoic acids were studied. Following intrauterine administration, the acidified uterine fluid was extracted with ether and back extracted into dilute NaOH, and their ultraviolet absorption and chromatographic characteristics were examined. Although m-hydroxybenzoic acid was recovered unchanged, p-hydroxybenzoic acid showed profound changes 48 hours after intrauterine instillation: a bathochromic shift of the absorption maximum from 282 mμ to 312 mμ and a change of Rf value in 2% acetic acid(8) from 0.85 for p-hydroxybenzoic acid to 0.94 for the product. While positive identification awaits further work, this absorption maximum is consistent with that of authentic 3, 5-dibromo-4-hydroxy-benzoic acid(9).

The minimal structural requirement for *in vivo* bromination was investigated with phenol, the simplest aromatic hydroxy compound. After intrauterine administration of phenol at pH 8 the uterine fluid exhibited an ultraviolet absorption maximum at 304 mμ as compared with 270 mμ for phenol and 302 mμ for 2, 6-dibromophenol. Unfortunately, paper chromatographic systems failed to afford well defined spots, making the bromination of phenol appear probable but not definite.

In 3 dogfish one uterus was filled with phenol red solution in sea water, and the other uterus of the same fish filled with an identical solution but with the addition of 10 mM of sodium azide. Bromination occurred only in the absence of sodium azide.

The source of bromine was studied in 4 dogfish. One uterus was drained, flushed twice with dogfish Ringer's solution(7) containing no bromide and then filled with a solution of phenol red in Ringer's solution. The other uterus was filled with a solution of phenol red in sea water. The uteri were then closed as described in Methods. After 24 hours the uterine fluids were withdrawn and inspected. Bromination occurred only in the uterus having access to bromide ions in the sea water. However, sea water as a bromide source is not essential because it could be replaced with a solution of NaBr. It therefore appears that the bromine is not derived from the fish but from an extraneous source.

Although the concentration of chloride ion in sea water is nearly 300 times as high as bromide, there was no indication of chlorination of any of the 4 dyes tested. To ascertain whether or not iodination would take place *in vivo,* solutions of sodium iodide, 0.85 mM (equivalent to the bromide concentration in sea water) in both sea water and Ringer's solution (without bromide) were separately instilled in different uteri of the same fish. After 48 hours, the fluid of both uteri turned dark blue. This resultant dye, however, showed absorption spectra identical with those of bromophenol blue. Also, both exhibited the same mobility in several paper chromatographic systems except in 0.58% ammonia. Further studies with I^{131} are planned to clarify this point.

Discussion. The bromination of phenol red by the pregnant dogfish uterus bears a close relation to the similar reaction catalyzed by

the chloroperoxidase system of *Caldariomyces fumago* except that the pH optima are different: 7.4 for dogfish uterus but 2.8 for the mold enzyme. Furthermore, addition to the incubation mixture of hydrogen peroxide, essential in the case of mold enzyme(4), is without noticeable effect on the bromination by dogfish uterus *in vitro*.

Bromination of phenol red, chlorophenol red, metacresol purple and fluorescein occurred after intrauterine administration of these dyes to the dogfish.

Evidence for the bromination of both phenol and p-hydroxybenzoic acid, although inconclusive, is suggestive. If enzymic bromination follows the generally accepted mechanism of aromatic bromination involving the electrophilic attack by a bromonium cation, one would expect the ready substitution of the hydrogens ortho to the hydroxyl and meta to the carboxylic group. The inertness of m-hydroxybenzoic acid would likewise be explained.

In the case of phenol red, as reported previously(3), the chromatogram of the uterine fluid exhibited an additional weak spot, besides that due to phenol blue. In the present investigation the paper chromatogram of uterine fluid of fish given fluorescein showed, in addition to eosin, spots whose Rf in 0.58% ammonia corresponded to synthetic 4,5-dibromo-, 2,4,5-tribromo-, and 2,4,7-tribromofluorescein(10). Also, when uterine fluid was collected after 24 hours instead of the usual 48 hours, the absorption maximum was 508 mμ as compared with 505 mμ for 4,5-dibromofluorescein. The present study does not give information as to which of the several isomeric monobromo-, and tribromo-fluoresceins was formed preferentially. However, available evidence suggests that bromination occurred stepwise.

The role of the bromination enzyme system in the physiology of the dogfish is obscure, and is being further investigated.

Summary. The observation that phenol red is brominated by the pregnant dogfish uterus *in vivo* was further studied and chlorophenol red, metacresol purple, and fluorescein were also found to be brominated. Some evidence was obtained that p-hydroxybenzoic acid and phenol were similarly brominated. The bromine was derived from an extraneous source. The site of bromination seemed to reside in the epithelium of the uterus. Bromination was also demonstrated by *in vitro* incubation, the pH optimum was found to be 7.4.

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