References and Notes

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5. Donated by Eli Lilly and Co., Greenfield, Ind., and by Merck and Co., Inc., Rahway, N.J.
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Bromination of Phenol Red by the Dogfish, Squalus acanthias

Abstract. The uterus of the pregnant spiny dogfish, *Squalus acanthias,* can convert phenol red into a new purplish-blue dye. Evidence shows that the new dye is bromophenol blue. This is the first example of biological bromination that has been observed in a vertebrate.

Apart from thyroxin and related substances, naturally occurring halogenated organic compounds are rare in animals. Certain species of shellfish, particularly *Murex brandaris* and *M. trunculus,* secrete a colorless liquid which, on exposure to light and air, gives the ancient dye, Tyrian purple, 6,6′-dibromoindigo (*1*). The occurrence of another bromocompound, 3,5-dibromotyrosine, has also been reported (*2*) in the skeletons of certain anthozoans. However, there appears to be no recorded instance of bromine being incorporated into an experimentally introduced exogenous material. The data presented in this report (*3*) indicate that the uterus of the pregnant spiny dogfish, *Squalus acanthias,* an elasmobranch, can convert the dye phenol red (phenolsulfonphthalein) into bromophenol blue (3,3′,5,5′-tetrabromophenolsulfonphthalein) (*4*).

The spiny dogfish has a gestation period of almost 2 years. In the first summer of pregnancy, the eggs in each uterus are enclosed in a common capsule, with the uterine wall in close apposition to the capsule. The capsule is broken, and in the second summer the embryos—small fish with yolk sacs, now called pups—lie free in the uterine cavity. The uterine fluid from the capsule stage (usually a few milliliters) has an electrolyte and urea content resembling that of elasmobranch blood. The uterine fluid of the pup stage, normally 50 to 100 ml, has no urea and has the same electrolyte composition as sea water, but with a *p*H of 5 to 6 (occasionally somewhat higher). The uterocloacal pore becomes flaccid, so that one can easily insert one's finger, and some uterine fluid is frequently lost when the fish is handled. Spontaneous intermittent emptying and filling of the uterus overnight was observed experimentally; this suggested that the sea water may gain direct entrance into the uterus. Whether the bromine used in the conversion of the phenol red comes from this uterine water or comes directly from the dogfish awaits further investigation.

In connection with a study on uterine function in the dogfish, phenol red, shown by paper chromatography to be free from any contaminating dyes, was dissolved in sea water, and 50- to 100-ml portions were introduced by funnel into the uteri of dogfish in the second summer of pregnancy. Saturated solutions as well as various dilutions of the dye were used. After 24 to 48 hours, all or most of the phenol red was replaced by a purplish blue dye, later identified as bromophenol blue. The conversion occurred without exception in each of the eight fish tested. Throughout the experiment, the dogfish were kept alive in sea water at 14° to 15°C.

Although the new dye (*X*) obtained from the uterine fluid (*p*H about 5 to 6) after the phenol red treatment is purplish blue in color, it soon turned yellow upon acidification. Further, the color change covered a *p*H range from 3.5 to 4.5, in agreement with that reported for bromophenol blue (*5*). The structural relationship between the two dyes suggests that this new dye may in fact be bromophenol blue or may be closely related to it. Chromatographic and light-absorption evidence supports the first possibility.

The uterine fluid containing the blue dye was acidified to about *p*H 1 with dilute HCl and extracted three times with benzene; each time about one half the volume of the aqueous phase was used. The benzene extract was washed with an equal volume of water and was then allowed to evaporate almost to dryness. The orange residue was finally recrystallized from methanol. Unfortunately the minute quantity of pure material did not permit a satisfactory ultimate analysis (*6*). Microfusion with sodium followed by micro- qualitative analysis did, however, result in a positive test for bromine and a negative test for iodine (*7*).

Chromatographic behavior in four solvent systems failed to show any significant difference between the blue dye *X* and bromophenol blue (Table 1). Additional faint spots of fluorescence were seen in two of the systems employed, as is indicated in the same table.

The light-absorption characteristics of the two dyes, in acidic and alkaline media, respectively, are listed in Table 2. The spectra of *X* and bromophenol blue were exactly superposable in the visible region, from 400 to 600 mμ. Between 220 and 360 mμ, the dye *X* demonstrated slightly stronger light absorption than bromophenol blue, but the maximal absorption of both occurred at exactly the same wavelength. In any event, the evidence at hand strongly suggests that the dye *X* and bromophenol blue are really one and the same.

No perceptible bromination of phenol red was observed under the following conditions: incubation of living or dead embryos with phenol red in sea water for 30 hours and incubation of phenol red with uterine fluid, blood plasma, and urine, respectively, for 72 hours at room temperature and also in the refrigerator at about 4°C. Uteri with embryos killed by asphyxia resulting from withdrawal of

Table 1. Comparison of the chromatographic behavior of the dye *X* and of authentic bromophenol blue (BPB). (Whatman No. 1 paper, ascending flow.) The spots indicated in the table appeared red under ultraviolet light in the 366-mμ region.

Compound	R_f			
	3% NaCl	BuOH-HOAc-H_2O (4:1:1)	BuOH-EtOH-H_2O (4:1:5)	BuOH saturated with 1.5*N* NH_4OH
X	0.65	0.76*	0.41†	0.41
BPB	0.61	0.76	0.44	0.42

* An additional faint spot of yellow fluorescence with R_f value 0.64 was also visible.
† An additional faint spot of yellow fluorescence with R_f value 0.61 was also visible.

Table 2. Optical densities (OD) of the dye *X* and bromophenol blue at their respective wavelengths of maximal absorption. The spectra were all determined in methanol. The concentration of bromophenol blue (BPB) was 10 μg/ml.

Compound	*p*H	λ_{max_1} (mμ)	OD	λ_{max_2} (mμ)	OD	λ_{max_3} (mμ)	OD
X	1.6	278	0.26	421	0.33		
BPB	1.6	278	0.17	421	0.33		
X	10.7	314	0.28	380	0.10	594	1.16
BPB	10.7	314	0.24	380	0.10	594	1.16

all uterine fluid 24 hours before infusion with phenol red did, however, effect bromination.

Further studies are in progress to determine the site and mechanism of the bromination. Perhaps there exists, in certain marine animals, an enzyme system which catalyzes the bromination described above. The biological halogenation of phenols may be analogous to the biogenesis of thyroxin where tyrosine residues undergo iodination (*8*).

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3. The biological phase of this work was aided by a grant from the New York Heart Association.
4. Bromophenol blue is prepared in the laboratory by reacting bromine with phenol red in glacial acetic acid. See E. C. White and S. F. Acree, *J. Am. Chem. Soc.* 41, 1190 (1919).
5. I. M. Kolthoff and V. A. Stenger, *Volumetric Analysis* (Interscience, New York, ed. 2, 1947), vol. 2, p. 53. It should be noted that phenol red itself changes color from yellow to red at *p*H 6.8 to 8.0.
6. A small amount (1.6 mg) of the crystalline material was nevertheless submitted for analysis for bromine; 37.06 percent was found. This seems to fit for bromophenol blue decahydrate; bromium content calculated for $C_{19}H_{10}Br_4O_5S \cdot 10\ H_2O$, is 37.60 percent. The microanalysis was performed by William C. Alford of the National Institute of Arthritis and Metabolic Diseases.
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6 October 1958

Ion Adsorption and Excitation

Abstract. A simple calculation shows that the K ions about to leave the nerve during excitation form a monolayer at the surface of the resting nerve and are not hydrated. The same applies to the Na ions which replace the K ions. This may be taken as an indication of the existence of a reversible ad- and desorption phenomenon.

An important ionic relationship associated with the excitation process becomes apparent in the following way. Let it be assumed that there is a specific volume V_K of the nerve substance in which the original concentration K_i of potassium ions changes to that of the bathing fluid K_o as a result of the net K ion loss incurred during a single discharge. This takes the form

$$V_K = K_{flux}/(K_i - K_o)$$

Knowing V_K and taking the flux quantity as distributed uniformly over the area of 1 cm², one can arrive at the thickness d_K of the nerve layer immediately involved in the exchange of K ions.

This simplified system is considered to be a valid one because of the steplike character of the K ion concentration gradient existing in the resting state between the two phases (that is, axoplasm and bathing solution). An additional advantage is the fact that the thickness thus obtained corresponds to that of a monoionic layer, obviating the necessity of considering a possible diffusion process which would tend to restore the internal K ion concentration to a uniform level. Similar reasoning holds with regard to d_{Na}.

The actual calculations were performed on the basis of the net ion flux data (K = 4.3 pmole/cm² per impulse, Na = 3.7 pmole/cm² per impulse) given by Keynes (*1*). The inner and outer ion concentrations used are those given by Keynes (*1*) for the *Sepia* axon (Na_i = 110 mmole/kg, Na_o = 458 mmole/kg, K_i = 272 mmole/kg, K_o = 9.7 mmole/kg), and those given by Hodgkin (*2*) for the *Loligo* axon (Na_i = 49 mmole/kg, Na_o = 440 mmole/kg, K_i = 410 mmole/kg, K_o = 22 mmole/kg).

The results are as follows, the figures in parentheses being values obtained from the second set of data.

$$d_K = 1.6393\ A\ (1.109\ A)$$
$$d_{Na} = 1.063\ A\ (0.946\ A)$$
$$d_K/d_{Na} = 1.54\ (1.172)$$

Examination of these results shows that the thickness of the nerve layer involved in ionic fluxes lies in the range of the effective radii of the ions considered (K = 1.33 A; Na = 0.98 A). In addition, the average of the two d_K/d_{Na} ratios obtained equals 1.367, which is very close to 1.357, the ratio of the effective radii of the K and Na ions.

It is thought that these results may justify the conclusion that the nerve layer immediately involved in ionic shifts allows for the presence of only one-half of a monolayer of nonhydrated cations. Accordingly, the original formulation is modified to take the form

$$f = A\ r\ (C_i - C_o)$$

where f is the net flux in moles per impulse, the sign denoting its direction; A is the area of the nerve in square centimeters; r is the effective radius of the ion in centimeters; and C_i and C_o are the inner and outer ion concentrations in moles per cubic centimeter.

Conversion of the concentrations into osmotic pressures shows the flux to be inversely proportional to the absolute temperature, a relation which is in line with the experimental evidence presented by Shanes (*3*).

Taking Hodgkin's data for plasma concentrations (*2*) and the flux values as above, one obtains from the flux formula K_i = 345.3 mmole/kg and Na_i = 62.45 mmole/kg. These values agree with those given by Koechlin (*4*) for the squid giant axons (K_i = 344 ± 15 mmole/kg, Na_i = 65 ± 15 mmole/kg), thus indicating a possible confirmation of the validity of the derived expression.

The emergence of the effective ionic radii implies that electrostatic forces may be at work and that the excitation phenomenon may involve to a greater or lesser degree an adsorption phenomenon (*5*).

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5. I thank Dr. G. Ungar and Dr. B. W. Zweifach for their advice and helpful criticism.

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Proliferation of Excised Juice Vesicles of Lemon in vitro

Abstract. Juice vesicles from mature lemon fruits will proliferate *in vitro* for indefinite periods. The comparatively simple tissue grows on a synthetic nutrient medium almost entirely inorganic in composition.

Many types of excised meristematic plant tissues or parts have been the subject of in vitro studies, but the successful culture of tissue from a mature fruit is mentioned only briefly in the literature. The first in vitro studies of fruit tissues were started by Schroeder (*1*) and are under way at present on several different fruit species (*2*).

In the present study mature lemon fruits (variety Eureka) were surface-sterilized by immersion for 20 minutes in a saturated calcium hypochlorite solution, rinsed with sterile water, and cut longitudinally into eighths. Removal of

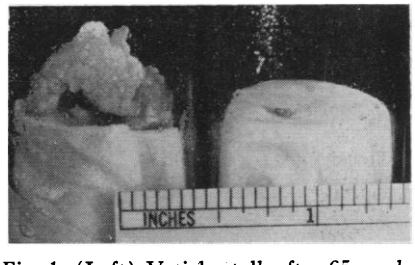

Fig. 1. (Left) Vesicle stalk after 65 weeks of in vitro growth. (Right) Dead vesicle stalk after 6 weeks of in vitro growth.