cubated overnight at 4°C with one of the two tracheal segments from each guinea pig; the other segment, serving as control tissue, was incubated under identical conditions, but without the antiserum. The mean relaxation of tracheal strips that had been incubated with antiserum to VIP was lower by 71 percent with 1-msec stimulations and 76 percent with 2-msec stimulations (Fig. 2).

Our results point to VIP as a transmitter of the nonadrenergic inhibitory nervous system in the airways of guinea pigs. Nerves containing VIP are widely distributed in organs with much smooth muscle, especially in sphincters (*15*). This peptide may also mediate enteric inhibitory responses (*16*), including the relaxation of the lower esophageal sphincter that is induced by electrical vagal stimulation.

Identification of the mediator of the nonadrenergic inhibitory system has obvious implications for the neurohumoral regulation of airway smooth muscle tone. Further, it is potentially important for understanding the pathogenesis of bronchial asthma and related bronchospastic disorders. Not only does the nonadrenergic inhibitory system appear to be the principal inhibitory nervous system for human airway smooth muscle (*2*), but a deficiency of this system could explain the hyperreactivity of airways in asthma (*17*). Support for this notion is provided by observations on Hirschsprung's disease, a condition characterized by persistent contraction of the distal bowel and localized absence of autonomic ganglia. In this disease, considered analogous in some respects to bronchial asthma (*17*), the nonadrenergic inhibitory responses are lacking (*18*) and VIP nerves are markedly reduced in affected areas of the gut (*19*).

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*p*H-Sensitive Liposomes: Possible Clinical Implications

Abstract. *When* p*H-sensitive molecules are incorporated into liposomes, drugs can be specifically released from these vesicles by a change of* p*H in the ambient serum. Liposomes containing the* p*H-sensitive lipid palmitoyl homocysteine (PHC) were constructed so that the greatest* p*H differential (6.0 to 7.4) of drug release was obtained near physiological temperature. Such liposomes could be useful clinically if they enable drugs to be targeted to areas of the body in which* p*H is less than physiological, such as primary tumors and metastases or sites of inflammation and infection.*

The use of liposomes as vehicles for preferential delivery of drugs to tumors has been discussed repeatedly (*1*–*4*). Despite the major effort invested in this idea, successful drug targeting is still an elusive goal. Recently, in an attempt to circumvent the targeting problem, it was proposed that local hyperthermia could preferentially release drugs from liposomes in the heated area (*5*, *6*). For this purpose, liposomes were constructed from phospholipid mixtures exhibiting gel-state to liquid crystalline phase transitions at a temperature slightly above body temperature and thus attainable by local hyperthermia.

In two studies in vivo, liposomes containing either radioactive methotrexate (*7*) or *cis*-dichlorodiamine platinum (PDD) (*8*) were injected into tumor-bearing mice. Local heating caused up to fourfold greater drug release and uptake by the tumor than in unheated areas. In addition, PDD specifically released from liposomes by heat produced a greater delay in tumor growth than did either free drug combined with tumor heating or drug containing liposomes but without local heating (*7*, *8*).

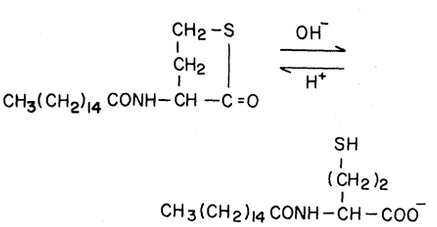

Fig. 1. Equilibrium between the uncharged thiolactone and charged open form of *N*-palmitoyl homocysteine (PHC). The thiolactone form is favored by decreasing *p*H.

The strategy of using local heating to induce preferential release of drugs from liposomes does not effectively address the major problem in human cancer: the metastatic lesions. An alternative approach was suggested by the fact that the interstitial fluids of a number of tumors in humans and animals have an ambient *p*H that is considerably lower than that of normal tissue (*9*–*12*). This difference could be utilized if liposomes could be constructed in a manner such that they would release encapsulated drug when passing through a region of lower *p*H. If microscopic domains of metastases also have a lower *p*H such vesicles could be of particular benefit in chemotherapy. In this report we describe *p*H-sensitive liposomes of possible therapeutic value.

Our approach to *p*H-sensitive lipo-

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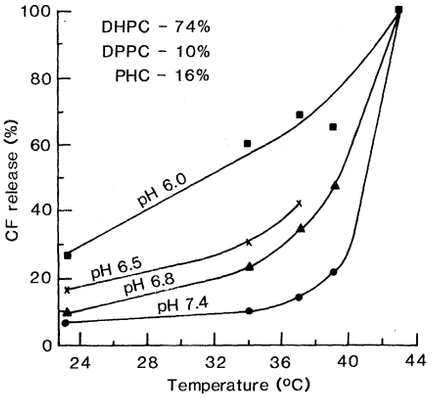

Fig. 2. Temperature profiles of CF release from liposomes at different pH's. Liposomes were prepared by sonication of lipids (2 mg/ml; 74 percent DHPC, 10 percent DPPC, 16 percent PHC) in 0.2*M* CF at pH 8.6 (0.1*M* carbonate buffer). The liposomes were separated from free CF on a Sephadex G-50 column and eluted with phosphate-buffered saline, pH 7.5. The CF at pH 8.6 was used for sonication in an attempt to ensure the maximum amount of PHC in the charged form. When the liposomes were run on the G-50 column, pH 7.5 buffer was used to bring them to a physiologic range. No CF loss was noted during the elution step. A liposome sample (20 to 30 μl) was added to 25 percent inactivated horse serum with different pH values (similar results are obtained with human or fetal calf serum), and the fluorescence intensity was measured at 541 nm (excitation at 465 nm) with a Perkin- Elmer 1000M fluorimeter at constant temperature. A rapid release of fluorescence was observed that plateaued within 5 seconds; this was followed by much slower (a few percent per minute) leakage. Values shown are at the 5-second time interval. The total fluorescence of each sample was measured by heating to 67° to 75°C, then cooling slowly to the assay temperature. This procedure takes into account the pH dependence of CF fluorescence and corrects for it (*23*). In 25 percent serum this procedure is consistent with release in the presence of detergent. Each symbol represents the average of two replicates.

somes is based on the incorporation of lipids with special head groups into vesicle walls composed primarily of dipalmitoyl, distearoyl, or diheptadecanoyl L-α-phosphatidylcholine (DPPC, DSPC, and DHPC, respectively). The internal aqueous compartment contained 0.2*M* carboxyfluorescein (CF), a self-quenched fluorescent marker whose release and dilution are accompanied by a marked increase in fluorescence yield (*13*). As the pH-sensitive lipid, we prepared *N*-palmitoyl L-homocysteine (PHC) (*14*, *15*). Free homocysteine exists in two forms, the open form and a thiolactone ring (*16*). The PHC could also be in equilibrium between open and closed forms, as illustrated in Fig. 1. In the open form, PHC resembles a free fatty acid and is well accommodated in lipid bilayers. In contrast, PHC thiolactone approximates a neutral lipid which destabilizes the bilayer, with a consequent drug release (*17*). The effect of serum pH and temperature on CF release was measured by the increase in fluorescence intensity (*18*) when portions of CF containing liposomes in pH 7.5 buffer were added to serum of different pH's. A temperature-dependent pH differential was obtained in serum with vesicles containing 12 percent PHC and 88 percent DPPC. The maximum differential in serum (pH 6.8 to 7.4) was obtained at 33°C, that is, below body temperature. Therefore, liposomes of differing composition were constructed. Addition of 1 to 5 percent DSPC gave little differential between 36° and 39°C and higher amounts resulted in unstable liposomes. Attempting to bring the temperature of optimal pH differential release closer to 37°C, we combined either 10 or 18 percent DPPC with DHPC. The drug-releasing characteristics of pH 7.5 liposomes are shown in Fig. 2, in which the curves represent typical temperature-release profiles at different pH values. When the pH of the test serum was lowered to either 6.5 or 6.0, a further increase in differential release was obtained.

Figure 3 summarizes CF release at different pH's in serum at 37°C for three liposome combinations. Only a minimum effect of pH on release of CF from pure DHPC vesicles at 37°C was noted (*19*), whereas, as expected from earlier studies (*5*, *6*), DPPC vesicles showed a high CF release in serum at 37°C. In contrast, the addition of PHC to mixtures of DHPC and DPPC produced a graded response that was inversely proportional to the serum pH. The amount of CF released at 37°C was approximately threefold greater at pH 6.5 than at 7.4 and fivefold higher at pH 6.0.

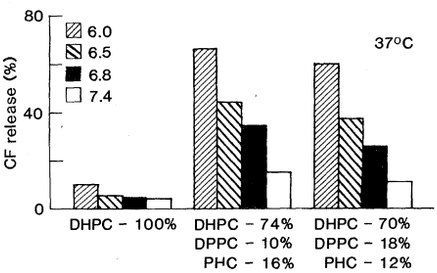

Fig. 3. Comparison of rapid release of CF from liposomes of three compositions, at 37°C and four different pH values. In the first set, with pure DHPC, each value is the average of two replicates. In the second set the data are from Fig. 2. In the third set, with 70 percent DHPC, 18 percent DPPC, and 12 percent PHC, we show averages of four replicates from two separate liposome preparations. We used the procedure as described in Fig. 2.

Plasma-induced leakage from liposomes, which occurs when the lipids are in the liquid crystalline phase (*20*), is unlikely because the time required is much longer (hours compared to seconds). Nevertheless, a less organized bilayer is required for maximum drug release.

The increased release of CF at low pH is probably not due to leakage resulting from conversion of CF to its nonionized form by H^+ ions, because the pK of CF ionization is more than two pH units below the range studied. At pH 6.0, more than 65 percent of the encapsulated CF was released from PHC-containing vesicles. In addition, little pH differential was observed in the DPPC-only liposomes (Fig. 3).

From the results we obtained with serum in vitro, we predict that drug release will be promoted if the liposome passes through a region of the body with a pH lower than physiological. A number of observations suggest that such conditions occur in vivo. For example, it has been demonstrated that the pH of efferent blood from rat Walker carcinoma 256 and hepatoma 5123 tumors have a lower average pH (as much as 0.18 unit) than afferent blood. The pH of tumor interstitial fluid is about 6.9 and can be further depressed by administration of either glucose or $NaHCO_3$, or CO_2 inhalation. In such cases the pH drops as low as 6.2. At the same time, the pH of the blood is either unchanged or slightly elevated (*9*, *10*). In regions of inflammation and infection, the exudate pH drops to 6.5, 60 hours after the start of the inflammatory reaction (*21*), and capillary permeability is increased in both tumors and inflamed sites.

If it proves possible to obtain specific release of drugs from pH-sensitive liposomes in animal tumors, such liposomes may become a clinically relevant means of drug delivery (*22*).

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18. A sample of lipids (DPPC, Fluka, Buchs, Switzerland; DHPC, Sigma Chemical Company, St. Louis, Mo.), at 2 mg/ml in 0.2*M* carboxyfluorescein (Kodak, recrystallized) at *p*H 8.6, was sonicated to clarity (15 minutes) at 70 W with a Branson B-12 Sonicator. The sample was chilled, centrifuged for 10 minutes to remove any metal fragments, and the liposomes obtained were separated on a Sephadex G-50 column at *p*H 7.5. The CF at *p*H 8.6 was used for sonication in an attempt to ensure the presence of the maximum amount of PHC in the charged form. When the liposomes were run on the G-50 column, *p*H 7.5 buffer was used to bring them to a physiologic range. No CF loss was noted during the elution step. The liposome fraction obtained was kept on ice and assays were started within 1 hour after preparation. After 24 hours at 4° to 6°C, some leakage of fluorescence could be detected, but not significantly more for liposomes containing PHC than for pure DPPC or DHPC vesicles.
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Developmental Equations for the Electroencephalogram

Abstract. *Thirty-two linear regression equations predict the frequency composition of the electroencephalogram within four frequency bands, for four bilateral regions of the brain, as a function of age. Equations based on such data from large groups of healthy children in the United States and Sweden are closely similar. These equations describe the development of the electrical activity of the normal human brain, independent of cultural, ethnic, socioeconomic, or sex factors.*

The frequency composition of the electroencephalogram, or EEG, reflects the age and the functional status of the brain. With maturation the dominant frequency becomes more rapid, and brain damage, dysfunction, or deterioration causes frequency slowing in the brain regions involved (*1*). These conclusions were initially based on qualitative impressions gained by visual examination of ink tracings. By means of analog filters and special-purpose frequency analyzers and, more recently, by using general-purpose digital computers implementing the fast Fourier transform (FFT), these conclusions have been confirmed by quantitative studies of changes in the EEG frequency spectrum with age and with brain disease (*2*).

The EEG frequency spectrum is considered to contain four major frequency bands: delta (1.5 to 3.5 Hz), theta (3.5 to

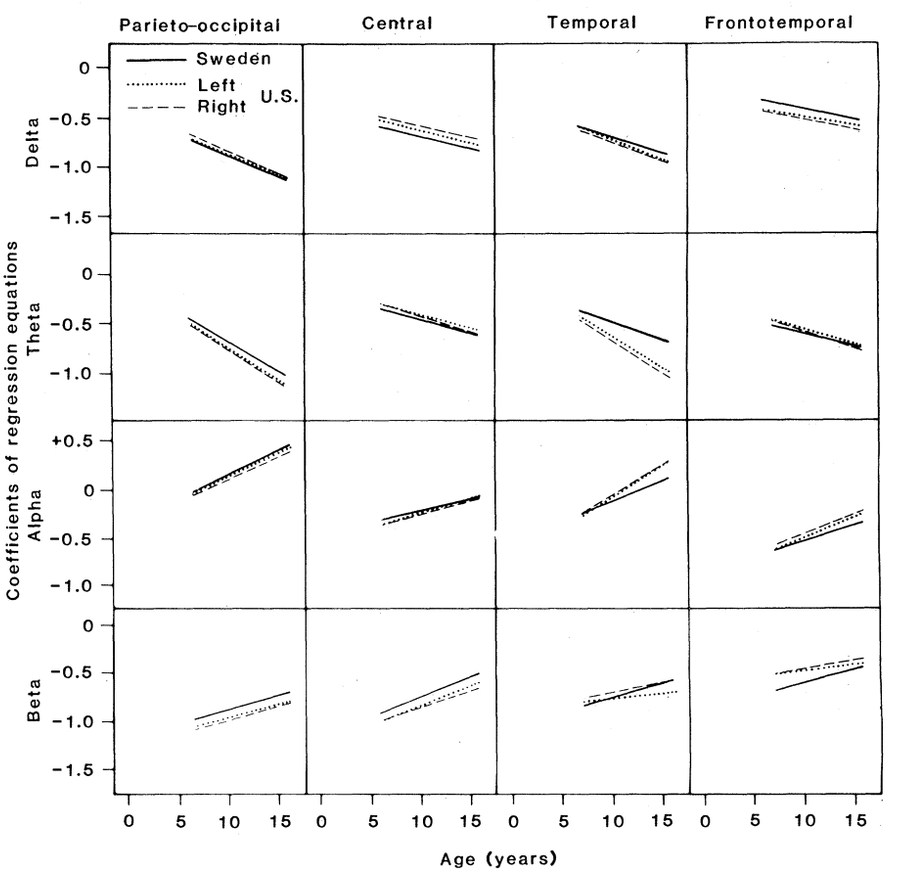

Fig. 1. Regression equations for data from U.S. children (N = 306) and Swedish children (N = 342) for each frequency band and derivation. Dashed lines (from right side of head) and dotted lines (left side) describe the equations derived from U.S. children. Solid lines describe the Swedish data. The data are valid for children aged 6 to 16 years.

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