dopamine incorporation (*9*). Synthetic ^{14}C-labeled NBAD was incorporated into isolated larval cuticle of *Calliphora erythrocephala*, which became light brown (*10*). Another β-alanyl catechol, *N*-β-alanylnoradrenaline, has been identified from the yellow wing pigments of the swallowtail butterfly, *Papilio xuthus* (*11*).

Our evidence points toward a central role for NBAD in the pupal tanning of *M. sexta* and in other insects that form brown cuticle in pupae, puparia, or adults. Its occurrence as a major catecholamine metabolite in several insect orders suggests that conjugation of dopamine with β-alanine may be as important as acetylation for sclerotization processes in insects. The type of cuticle formed and its coloration, however, may depend on which form of dopamine is produced.

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2. *Manduca sexta* were reared and tissues were collected as described [K. J. Kramer, T. L. Hopkins, R. F. Ahmed, D. Mueller, G. Lockhart, *Arch. Biochem. Biophys.* **205**, 146 (1980)]. Hemolymph was homogenized in ice-cold 1.2*M* HCl, 1:9 (by volume), containing 4.8 m*M* sodium bisulfite and the internal standard, dihydroxybenzylamine, and centrifuged at 6500*g* for 10 minutes. The catechols were adsorbed from the supernatant on alumina and analyzed by LCEC [L. L. Murdock and D. Omar, *Insect Biochem.* **11**, 161 (1981)]. Mobile phase and operating conditions are described in Fig. 1. Portions of the supernatants were also hydrolyzed in 1.2*N* HCl at 100°C for 10 minutes to release conjugated catechols before alumina adsorption and LCEC.
3. Samples of compound V isolated by liquid chromatography and desalted on a Biogel P-2 column were hydrolyzed at reduced pressure in 6*N* HCl for 2 hours and 24 hours. Analysis of the constituent amino acids by liquid chromatography as their fluorescent *o*-phthaldialdehyde derivatives [D. W. Hill, *et al.*, *Anal. Biochem.* **51**, 1338 (1979)] gave a single peak eluting at the retention time of β-alanine (30.5 minutes). Analysis of the 6*N* HCl hydrolyzate by LCEC showed the disappearance of compound V and the appearance of dopamine.
4. Samples were hydrolyzed at reduced pressure in 2*N* HCl for 2 hours, silylated, and analyzed by gas chromatography [R. A. Wirtz and T. L. Hopkins, *J. Insect Physiol.* **20**, 1143 (1974)]. The retention times for silylated β-alanine and dopamine were 2 and 23 minutes, respectively, on a 5 percent OV-1 column with a column temperature of 200°C and a carrier gas flow rate of 84 ml/min.
5. Synthetic NBAD was prepared by coupling dopamine with *N*-α-*t*-butyloxycarbonyl-β-alanine-*N*-hydroxysuccinimide ester in potassium tetraborate buffer and deblocking the conjugate in 0.1*N* HCl. Identity was confirmed by LCEC and ultraviolet spectroscopy. Purity was > 99 percent.
6. Concentrations of hemolymph NBAD and *N*-acetyldopamine, respectively, for A, white adults; L, mature larvae; and P, white pupae or puparia were: *Periplaneta americana* (A), 91 and 63 μ*M*; *Sarcophaga bullata* (P), 254 and 53 μ*M*; *Diatraea grandiosella* (P), 310 and 10 μ*M*; and *Thyridopteryx ephemeraeformis* (L), 80 and 22 μ*M*. Whole body concentrations were *Plodia interpunctella* (P), 147 and 168 μmole/g; *Ephestia cautella* (P), 87 and 8 μmole/g; and *Tenebrio molitor* (P), 41 and 77 μmole/g.
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8. *o*-Diphenol oxidase was extracted from pharate pupal cuticle, precipitated with 30 percent ammonium sulfate and chromatographed on Sephacryl S-300 in 0.1*M* ammonium bicarbonate, *p*H 8.5. Activity was determined spectrophotometrically at 470 nm (dopamine) and 390 nm (*N*-acetyldopamine and NBAD). Velocity data were treated by Lineweaver-Burk and nonlinear least-squares analyses.
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13. We thank R. F. Ahmed for gas-liquid chromatography analysis and G. Lookhart for β-alanine analysis by liquid chromatography of some hydrolyzates. This is contribution No. 82-157-j from the Departments of Entomology and Biochemistry, Kansas Agricultural Experiment Station, Manhattan 66506. Cooperative investigations of Agriculture Research Service, Department of Agriculture, and the Kansas Agricultural Experiment Station. Supported in part by research grant PCM-8003859 from the National Science Foundation.

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Size Analysis of Phospholipid Vesicle Preparations

Abstract. *Gel exclusion chromatography based on the use of Sephacryl S-1000 provides a quick and convenient method for determining the average diameter of phospholipid vesicles and an approximate measure of size heterogeneity.*

Recently, Kirkland *et al.* (*1*) pointed out the inconvenience associated with the use of electron microscopy for routine size analysis of phospholipid vesicle dispersions and demonstrated the applicability of the technique of sedimentation field flow fractionation to this problem. Since this is a separation method, it can be used not only for analytical purposes but also for the preparation of nearly homogeneous fractions from initially polydisperse mixtures. We report here that gel exclusion chromatography can also be used for both these purposes. This was one of the methods used by Huang (*2*) in his study of vesicle size. The vesicles prepared by Huang were very small (diameter < 25 nm), and the gel filtration media available at the time could not have handled particles much larger. Materials with larger pore size have been developed since then, and with Sephacryl S-1000 (Pharmacia, Inc., Piscataway, New Jersey) the size analysis has been extended to a diameter of about 300 nm. Much current research focuses on single-walled vesicles, which generally have diameters below this limit. For many practical projects, such as the use of phospholipid vesicles for drug delivery, it is necessary to have a reasonably accurate measure of average vesicle size but only a semiquantitative measure of polydispersity (*1*). Gel chromatography with Sephacryl S-1000 adequately meets these requirements.

The results reported here were obtained with single-walled vesicles prepared by detergent removal from detergent-solubilized egg yolk phosphatidylcholine. Mimms *et al.* (*3*) have described a preparative method in which octyl glucoside was used as solubilizing detergent, and either dialysis or gel chromatography (on a small-pore resin) was used for detergent removal. The data

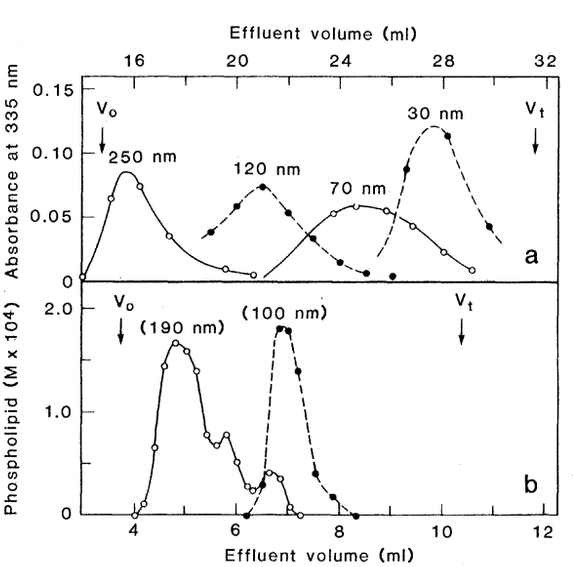

Fig. 1. (a) Elution patterns for four vesicle preparations, each determined separately on column A. A different detergent was used for each preparation: from left to right they were octyl glucoside (*2*), dodecyl octaethylene glycol monoether (*8*), polyoxyethylene 9 lauryl ether (Sigma Chemical Corporation, St. Louis), and sodium cholate (*8*). (b) Elution patterns on column B for two preparations, both made with octyl glucoside as the solubilizing detergent but differing in the manner and rate of detergent removal.

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presented here are an extension of this procedure, in which different detergents and several methods of detergent removal were used. Many of the preparations were examined by electron microscopy to determine a nominal average diameter (calibration of the gel filtration medium was one of the objectives), and chloride permeability measurements (*3*) were used to demonstrate the integrity of the vesicle membranes and the existence of a trapped volume of appropriate magnitude.

Two kinds of column were used, and both were presaturated with phospholipid to avoid adsorption during chromatography. Polystrene latex particles (Polysciences, Inc., Warrington, Pennsylvania) or multilamellar lipid vesicles were found to be suitable as void volume markers. Column A was designed for cursory examination of freshly prepared samples and was intended to provide (if necessary) a final fractionation step in the preparative procedure. The column dimensions were 50 by 0.9 cm; the void volume (V_0) was 14.8 ml; and the total volume (V_t) was 31.6 ml. The flow rate was 3 ml/hour, sample size was about 1 ml, and fractions of 0.5 to 1 ml were collected and analyzed. Column B was intended as a purely analytical column. The dimensions were 28 by 0.7 cm, $V_t - V_0$ was 6.7 ml, flow rate was 3 ml/hour, sample size was 0.2 ml, and the collected fraction size was 0.15 to 0.2 ml.

Figure 1a shows elution profiles obtained with four separate vesicle preparations on column A. Turbidity (absorbance at 335 nm) was used as a measure of vesicle concentration. Nominal diameters obtained by electron microscopy are shown for each sample.

Figure 1b shows elution patterns obtained with column B, in an experiment designed to investigate the effect of different methods and rates of detergent removal from initially similar lipid-detergent solutions. Organic phosphorus analysis (*4*) was used as a measure of lipid concentration for all fractions with significant absorbance at 230 nm (measured in a microcell). The data for column B show that multimodal size distributions are obtained under some conditions and are readily detected by this technique. This column was not calibrated: diameters shown for the principal peaks are based on the assumption that the calibration data for column A are applicable.

In order to show that the chromatographic behavior of Sephacryl S-1000 is similar to that of smaller pore resins, we have used the nominal average diameters obtained by electron microscopy and the peak positions of Fig. 1a to generate a

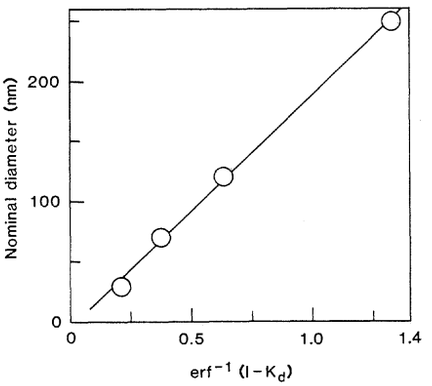

Fig. 2. Plot of the peak positions of Fig. 1 according to the equation of Ackers (*5*).

plot of the data according to the inverse error function (erf^{-1}) equation of Ackers (*5*). For spherical vesicles, the diameter should be precisely twice the Stokes radius, and Fig. 2 demonstrates that the linear relation between diameter and erf^{-1} $(1 - K_d)$ that is typical of earlier work with much smaller particles (*5*) applies here as well (*6*).

These results demonstrate the utility of gel exclusion chromatography as a quick and convenient technique for characterizing vesicle preparations. Provided that the eluting buffer and the internal vesicle solution are approximately isoosmotic, the technique can be applied to vesicles containing any desired trapped solute. Because sample size, flow rate, diffusion, and column dimensions influence the spread of an elution peak in a rather complex way (*7*), the chromatographic method is not well designed for a rigorous determination of particle size distribution. The narrow width for the second curve in Fig. 1b, however, suggests that size heterogeneity is the major determining factor (there is no reason to believe that the sample is truly monodisperse), so that peak width can be treated with confidence as at least a relative measure of polydispersity.

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Immunotherapy of Metastases Enhances Subsequent Chemotherapy

Abstract. *In many multimodal therapies of cancer, postsurgical chemotherapy is administered before immunotherapy for treatment of micrometastatic disease. This sequence may not be the most efficacious. Experiments in which strain 2 guinea pigs bearing syngeneic L10 hepatocarcinomas were given immunotherapy showed that infiltrating immune effector cells not only were tumoricidal but disrupted the characteristically compact structure of metastatic foci. When cytotoxic drugs were administered at the peak of this inflammatory response, the survival rate of the guinea pigs increased significantly. We conclude that postsurgical immunotherapy can enhance the effect of cytotoxic drugs administered subsequently.*

Considerable effort has been devoted to understanding how tumors avoid elimination by immunotherapy. Many mechanisms have been proposed, most of which attribute treatment failures to a compromised immune status of the host or a lack of tumor immunogenicity (*1*). Relatively little attention has been given to the possibility that anatomic characteristics of the metastases serve to protect them from therapy. Solid tumors as small as 1 mm in diameter contain areas of severe vascular insufficiency (*2*). These areas contain many viable but hypoxic cells. Even in culture, tumor cells grown as spheroids show evidence of hypoxia when the colony is larger than 0.35 mm in diameter (*3*). Thus, it is probable that most occult metastases have vascular insufficiencies and hypoxic cells, factors that could make metastases resistant to cytotoxic drugs or infil-

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