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Development of a System for Controlled Release of Benzo(*a*)pyrene, 7,12-Dimethylbenz(*a*)anthracene, and Phorbol Ester for Tumor Induction in Heterotopic Tracheal Grafts[1]

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ABSTRACT

The utility of the tracheal transplant model as a tool in *in vivo* carcinogenesis studies depends largely on the development of a good drug delivery system affording reproducible, sustained carcinogen release. Previously used methods have proven to be less than satisfactory.

We studied the release of benzo(*a*)pyrene, 7,12-dimethylbenz(*a*)anthracene, and 12-*O*-tetradecanoylphorbol-13-acetate from pellets of varying composition. *In vitro* release of the two polycyclic hydrocarbons (PCH) from beeswax pellets showed little variability, regardless of PCH concentration. In marked contrast, *in vivo* release was highly variable, particularly at high PCH concentrations. This suggested that the *in vivo* variability was largely due to the toxic alterations of tissues, caused by carcinogen released at high rates.

Pellets composed of beeswax:cholesterol in ratios of 1:1 to 1:9 showed markedly reduced rates of PCH release. At a ratio of 1:9, the overall release rate of benzo(*a*)pyrene was ~1 μg/day compared to ~7 μg/day for pellets with a pure beeswax matrix [100 μg benzo(*a*)pyrene pellets]. The variability of PCH release was simultaneously diminished, supporting the suspicion that it was a result of toxic tissue changes. Similarly reduced release rates could be obtained by adsorbing the PCH to charcoal particles.

Studies with the promotor 12-*O*-tetradecanoylphorbol-13-acetate showed a release rate of 1.6 μg/day from pure beeswax (100 μg 12-*O*-tetradecanoylphorbol-13-acetate per pellet). This rate may have to be diminished before promotion studies in the tracheal transplant model can be attempted. Our studies demonstrate that protracted PCH release from pellets can be achieved by using a beeswax: cholesterol matrix instead of a pure beeswax matrix or by adsorbing the PCH to charcoal particles. Such pellets release at fairly constant rates, with little pellet-to-pellet variability. Thus, the major problem of using the tracheal transplant model for quantitative tumor induction studies with PCH's appears to be resolved.

INTRODUCTION

We recently described the heterotopic tracheal transplant model as a new experimental system to study the development of neoplastic disease in respiratory tract epithelium (6). We pointed out that this experimental model lends itself particularly well to investigation of the role of various host and environmental factors as modifiers of respiratory tumorigens since host and target organ can be independently manipulated and since initiators and carcinogens as well as promotors and cocarcinogens can be applied in known quantities to a narrowly defined target site. In this system the mucosa of s.c. tracheal transplants is exposed directly to test substances contained in and released from an intraluminal, rod-shaped pellet. We have described the induction of neoplasms with several types of carcinogenic polycyclic hydrocarbons, the carcinogen dose-tumor response relationships, and the time-dependent appearance of various presumed preneoplastic lesions following PCH[5] exposure (3, 4, 10). Since our first report of this experimental model (6), other laboratories have also used it with equal success (7, 8).

During our studies over the last 2 years, it became increasingly clear that the ultimate utility of this experimental model depended largely on the development of a "drug delivery system" allowing protracted exposure at reproducible, fairly constant and controllable rates with a variety of chemicals. Clearly, it was necessary to investigate this component of the experimental model in greater detail than we had previously done. In the initial studies (6) the PCH's had been incorporated into pellets composed of a gelatin matrix that rapidly dissolved on introduction into the tracheal lumen, releasing the total carcinogen dose within 5 to 7 min. Subsequently, pellets with a beeswax matrix were found to release the carcinogen more gradually (2, 4, 10). However, inconsistencies in PCH release rates in various studies prompted us to study the source of this variability and to look for means to correct this deficiency.

The purpose of this report is to present our efforts at identifying the cause of the variability in the carcinogen delivery system used to induce tracheal tumors and to find means to reduce or eliminate it. *In vitro* and *in vivo* studies of the release characteristics of PCH's from beeswax pellets containing high and low concentrations of PCH's are described. The *in vitro* studies were performed because of the speed of the assay and for investigation of PCH release independent of confounding biological variables. We report

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[5] The abbreviations used are: PCH, polycyclic hydrocarbon; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; BP, benzo(*a*)pyrene; DMBA, 7,12-dimethylbenz(*a*)anthracene; i.t., intratracheal.

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modifications of the pellet matrix, which, dependent on the pellet composition, effect markedly slower and more consistent rates of PCH release than those obtained previously with a pure beeswax matrix. Finally, we describe release experiments with the classical promoting agent, TPA.

These studies may be of interest not only to the student of respiratory tract carcinogenesis but also to investigators interested in protracted release of PCH's and TPA in other *in vivo* models of carcinogenesis.

MATERIALS AND METHODS

Animals

Ten-week-old isogenic Fischer 344 rats were bred and reared in an animal isolation facility. These animals were demonstrated to be free of the common infectious diseases of rats, including pneumonia.

Chemicals

White (USP grade) beeswax was obtained from Fisher Scientific Co., Pittsburgh, Pa. Molton wax was purified by suction filtration through a steam-jacketed funnel with a medium-porosity, fritted glass disc (pore diameter, 10 to 15 μm). BP (99+%; gold label) and cholesterol (98%) were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and used without any further purification. DMBA was obtained from Eastman Organic Chemicals, Rochester, N. Y., and was purified as described below. TPA and [^{3}H]TPA (2 Ci/mmol) were purchased from P. Borchert, University of Minnesota, Minneapolis, Minn. NCS, [^{14}C]BP (specific activity, 25 mCi/mmol), and [^{14}C]DMBA (21.2 mCi/mmol) were obtained from Amersham/Searle Corp., Arlington Heights, Ill. The last 2 chemicals were purified on silica gel plates in a solvent system of benzene:*n*-hexane (1:15; Solvent A). Heat-inactivated fetal calf serum, human serum, and rat serum were obtained from Grand Island Biological Co., Grand Island, N. Y. PPO (scintillation grade) was received from Packard Instrument Co., Inc., Downers Grove, Ill. Carbon dust (5 to 10 μm; prepared from activated charcoal supplied by Barnebey-Cheney Co., Columbus, Ohio) was obtained through the courtesy of Jean Graf, IIT Research Institute, Chicago, Ill. Silica gel plates were purchased from Brinkmann Instruments, Inc., Westbury, N. Y. Silastic materials were obtained through the courtesy of Dow Corning Corp., Midland, Mich. All other chemicals were reagent grade.

Purification of DMBA

A suspension of DMBA (1 g in 200 ml 99% ethanol) was heated on a steam bath for 15 min in a 500-ml, round-bottomed flask fitted with a reflux condenser. The mixture was filtered hot through Celite with a Büchner funnel. The clear yellow filtrate was stirred magnetically at 23° with 0.5 g Norit A decolorizing carbon for 20 min. The mixture was filtered through a fluted filter paper; exposure to light was avoided as much as possible in this and all subsequent steps. The almost colorless filtrate was evaporated to dryness in a rotary evaporator at 40° under reduced pressure (20 mm), and the residue was dissolved in 60 ml of boiling 99% ethanol and allowed to crystallize by cooling in ice. After 2 hr the crystals were collected by filtration, dried in a vacuum oven at 40° for 3 hr, and stored in amber-colored bottles over nitrogen in a refrigerator. The yield of DMBA was 340 mg. A good second crop of crystals (240 mg) was obtained from the mother liquor. The combined yield was 580 mg (58%), m.p. 123–123.5° [literature, 122.4–122.8° (11)], and crystals were chromatographically homogeneous by thin-layer chromatography in Solvent A. λ_{max} (methanol), 295 nm (ϵ = 77,800), 283.5 nm (ϵ = 66,730); λ_{max} (benzene), 301 nm (ϵ = 79,000), 288.5 nm (ϵ = 61,900). Previously published spectral data were: λ_{max} (methanol), 295.5 nm (ϵ = 90,100), 285 nm (ϵ = 77,300) (Ref.16); λ_{max} (ethanol), 296.5 nm (ϵ = 79,433), 285 nm (ϵ = 63,096) (Ref. 13). Our data tend to agree with those reported in Ref. 13 and to differ from those reported in Ref. 16. Short-path cuvets (2 mm) used in the measurements reported in Ref. 16 may have partly contributed to the error.

Assay of BP, DMBA, and TPA

Spectrophotometry. Beeswax pellets containing BP or DMBA were dissolved in benzene. Beeswax pellets containing carbon particles coated with BP were extracted with benzene for 2 hr in a Soxhlet apparatus with double-thickness cellulose thimbles. The following molar extinction coefficients were used for determining the concentrations of different carcinogens in benzene: for BP, λ_{max} (benzene), 388 nm (ϵ = 30,960); for DMBA, λ_{max} (benzene), 301 nm (ϵ = 79,000). Beeswax or benzene does not interfere with spectrophotometric assay at these wavelengths.

Radioassay. Concentrations of [^{3}H]TPA, [^{14}C]BP, and [^{14}C]DMBA in fetal calf serum were assayed by dissolving 0.2-ml aliquots of serum in 1 ml NCS followed by addition of 10 ml scintillation cocktail (0.5% PPO in toluene) and counting in a Beckman LS-233 liquid scintillation counter. All 3 compounds had a specific activity of approximately 2000 dpm/μg.

Preparation of Pellets

Beeswax. Pellets were prepared as described previously (2), with the following modifications. Weighed amounts of carcinogen (a Cahn Electrobalance was used for weighing mg amounts) and beeswax were melted in a scintillation vial that was heated in a cavity in an aluminum block placed on a hot plate with thermostatic control. The aluminum block had a thermometer placed in a central hole to monitor the temperature. A clear melt was obtained with DMBA and BP at 80°. The PCH:beeswax mixture was assayed to determine the PCH concentration obtained and to adjust it, if necessary, before making pellets. An appropriate aliquot of the mixture was transferred to the preheated mold for pressing out the pellets (14.8 mm long x 1.5 mm in diameter). PCH:cholesteryl laurate:beeswax pellets were made the same way. The average weight of plain beeswax pellets was 24.48 mg ±0.62 (S.D.; 95% confidence range, 23.24 to 25.72). Pellets containing PCH were individually weighed, and those that did not fall within ±2.0% of the mean pellet weight were rejected. Thus, a pellet labeled as 100 μg BP: beeswax actually contained 98 to 102 μg BP. We had about

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30% rejections for every batch of pellets processed with the pellet maker.

PCH:Cholesterol:Beeswax. The PCH:cholesterol:beeswax mixture was heated to 160° in a scintillation vial (as described previously for beeswax) and in a nitrogen atmosphere to prevent oxidation of PCH's and to form a clear melt and was then made into pellets (as described previously). Since these pellets were fragile, particularly at high concentrations of cholesterol, they were encased in Dow Corning Silastic tubing (medical grade; Catalog No. 602-234; inside diameter, 1.47 mm; outside diameter, 1.96 mm) and sealed at the ends with Silastic type A medical adhesive silicone (Catalog No. 891). Results of *in vitro* experiments (not shown) with both bare and Silastic-enclosed PCH: beeswax pellets indicated that Silastic did not influence the release rates significantly.

BP Coating of Carbon Particles. Carbon particles 5 to 10 μm in diameter were internally coated by heating and stirring with BP in acetone solution as described in Report No. IITRI-C6184 of June 1970, under Contract NIH-90-0275. Carbon was then incorporated in the beeswax pellets.

Carbon particles (2.0 g) were added with stirring to a hot solution of BP (2.2 g) in acetone in a beaker. Acetone was allowed to evaporate overnight in the hood at room temperature. All of the dried, caked mixture from the walls of the beaker was scraped down, and the side of the beaker was rinsed with 1 ml acetone. The mixture was heated gently with constant stirring and scraping. After all of the acetone was evaporated, the mixture was heated to about 200°, with continued stirring and scraping. As the BP melted the mixture became sticky; it went dry again in 10 sec when the molten BP was adsorbed onto the carbon. After the mixture became dry and powdery, it was heated for about 1 min at which time white vapors began to appear. The final product was cooled in a desiccator. The BP content was assayed by extracting a weighed portion of the sample for 2 hr with benzene in a Soxhlet extractor with a double-thickness cellulose thimble. The BP concentration in benzene extract was determined spectrophotometrically. The BP-coated carbon was then incorporated in the beeswax pellets.

Procedure for Release Experiments

In Vitro. Carcinogen pellets were shaken in a 25-ml Erlenmeyer flask with 6 ml fetal calf serum at 37° in a water bath shaker at 100 rpm (Model G86; New Brunswick Scientific Co., New Brunswick, N. J.). Serum was changed at 24-hr intervals, and the amount of released carcinogen was determined by radioassay. Sterile conditions were observed as far as practicable.

In Vivo. Pellets containing a known amount of carcinogen were inserted into established tracheal grafts as described by Kendrick *et al.* (6) and as shown in Fig. 1. The pellets were recovered at certain time intervals, and the remaining carcinogen content was determined by either spectrophotometry or radioactivity.

Solubility of BP

In Serum. Four ml serum were shaken in a stoppered 10-ml Erlenmeyer flask in a water bath shaker at 37° and 100 rpm with approximately 20 mg [^{14}C]BP (specific activity, 2500 dpm/μg). At certain intervals, 0.4-ml aliquots were withdrawn and filtered under pressure through a fine-fritted glass disc (pore diameter, 4 to 5.5 μm). A 0.2-ml aliquot of the filtrate was counted for radioactivity. The filtrate was also counted for radioactivity after centrifugation at 250 × *g* for 30 min to rule out the presence of fine BP particles. Practically no difference was observed in the radioactivity of the filtrate before and after centrifugation. Samples were taken and counted until the concentration of BP in the serum leveled off. These experiments were repeated with a Millipore syringe filter (Catalog No. XX3001200) and a filter with an average porosity of 0.22 μm (Catalog No. GSWP-01300), with essentially similar results.

In Beeswax. Weighed amounts of BP and beeswax were heated together until the mixture formed a clear homogeneous melt; it was then transferred into the mold, and 5-μm-thick sections were cut with a microtome. In this way sections were prepared from the BP:beeswax mixture and contained 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 4% (w/w) BP. The sections were studied and photographed at 37° under a Zeiss UV epifluorescence microscope (UV source, halogen bulb; excitation filter, KP490-500; barrier filter, LP528). The maximum concentration at which BP crystals were not discernible under the microscope was taken as the solubility of BP in beeswax at 37°.

RESULTS

Solubility of BP in Different Sera and Beeswax. Since we had decided to use serum as the medium for the *in vitro* experiments, it was important to determine the solubility of the PCH's in different sera. Solubility was lowest in fetal calf serum (35 μg/ml) and highest in human serum (214 μg/ml), as shown in Table 1. Obrikat and Wettig (12) reported the solubility of BP in human serum as 50 μg/ml. This discrepancy is presumably due to the difference in the temperature at which solubility was determined. We carried out our determination at 37°, whereas Obrikat and Wettig probably determined the solubility at room temperature since no temperature is mentioned in their paper. Our data were reproducible with different techniques (see "Materials and Methods"). For all subsequent *in vitro* studies, fetal calf serum was chosen as medium because of the availability of a large amount from the same batch. The solubility of BP in beeswax was tested by mixing BP powder with melted beeswax at various concentrations and allowing the beeswax to solidify. Sections were then prepared from the beeswax with a microtome and were studied with the microscope under UV at 37°. We found that, at a concentration of approximately 0.5% (w/w) and below, no crystalline BP could be observed at a magnification of ×400, whereas at higher concentrations BP crystals of various sizes could be discerned. We took this to indicate that the BP solubility in beeswax was approximately 0.5% at 37°. The 100-μg BP:beeswax pellets containing 0.41% (w/w) BP did not show BP crystals (Fig. 2*A*). In contrast, the 1000-μg BP pellets containing 4.10% (w/w) BP showed many large and small crystals (see Fig. 2*B*).

***In Vitro* and *In Vivo* Release of BP and DMBA from Beeswax Pellets.** We wanted to obtain detailed information

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Table 1
Solubilities of BP in different sera at 37°

Serum[a]	Equilibrium solubility (μg/ml)
Fetal calf	35.6 ± 6.9[b] (3)[c]
Fasted rat	131.6 ± 3.2 (3)
Unfasted rat	147.4 ± 9.4 (4)
Human	214.6 ± 13.6[d]

[a] Fetal calf serum (Batch C-962918) and human serum (Batch C-467124) were obtained from Grand Island Biological. Unfasted and fasted rat serum were obtained from Fischer 344 rats in the laboratory.
[b] Mean ± S.D.
[c] Numbers in parentheses, number of measurements.
[d] Reported 50 μg/ml at room temperature (12).

on the characteristics of release kinetics of the 2 major PCH's used in the tracheal transplant studies. It seemed likely that some type of feedback between carcinogen release and the resulting tissue changes would develop. We therefore investigated PCH release *in vitro* as well as *in vivo*. NaCl solution (0.9%) or pseudoextracellular fluid containing physiological concentrations of Na^+, K^+, Cl^-, bicarbonate, and phosphate has been used as the release medium by previous investigators (5, 15, 17). Since PCH's are practically insoluble in aqueous medium, we chose to use fetal calf serum, which, with its protein and lipid components, more closely resembles the extracellular fluids surrounding the pellets *in vivo*. That we might keep it sufficiently below the saturation level of 35 μg/ml of serum, the medium was changed every 12 to 24 hr (6 ml medium are used in the *in vitro* test; *i.e.*, saturation would occur at 6 × 35.6 μg BP). This schedule of serum changes created a situation resembling an infinite sink not much different from the continuous flow system used by Roseman and Higuchi (14).

Release rates were studied at 2 PCH concentrations, namely, 0.41 and 4.10% (*i.e.*, 100 and 1000 μg PCH per pellet). These concentrations were chosen because according to previous studies (4, 10) they appear most useful for tumor induction with DMBA and BP. The findings are presented in Charts 1 and 2 and can be summarized as follows.

1. Release rates *in vitro* and *in vivo* are concentration dependent; *i.e.*, as the concentration in the pellets diminishes with time, the amount released per unit of time diminishes (a typical example is illustrated in Chart 1*A*).

2. The rate of *in vivo* release from pellets containing 100 μg PCH is faster (100% released in 2 weeks) than that from pellets containing 1000 μg PCH (100% released in >4 weeks). Most likely, this is due to the undissolved carcinogen present in the pellets containing 1000 μg PCH (*cf.* Charts 1, *A* with *B*, and 2, *A* with *B*, and Fig. 2, *A* with *B*).

3. At low concentrations of PCH, *in vitro* and *in vivo*, release rates are similar (see Charts 1*A* and 2*A*); the latter are slightly slower. At high PCH concentrations, the *in vivo* release rates are markedly slower than those seen *in vitro* (see Charts 1*B* and 2*B*).

4. *In vitro* release rates of PCH show only a minor degree of variability at low and high concentrations; the maximum percentages of S.D. are 18 and 23, respectively. In contrast, *in vivo* variability is high and is particularly pronounced at high PCH concentrations; maximum percentages of S.D. are 45 and 96 at low and high concentrations, respectively.

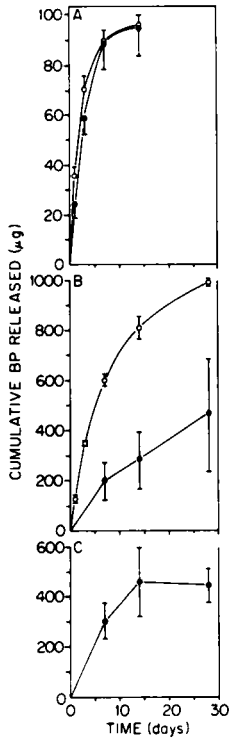

Chart 1. Cumulative release of BP from beeswax pellets. *A*, pellets containing 100 μg BP. ○, *in vitro* release; ●, *in vivo* release in tracheal grafts. Each *point* represents the mean observation from 4 pellets *in vitro* ± S.D. and 6 pellets *in vivo* ± S.D. *B*, pellets containing 1000 μg BP. ○, *in vitro* release; ●, *in vivo* release in tracheal grafts. Each *point* represents the mean observation from 3 pellets *in vitro* ± S.D. and 12 pellets *in vivo* ± S.D. *C*, pellets containing 1000 μg BP implanted s.c. Each *point* represents the mean observation from 6 pellets ± S.D.

$$\% \text{ of S.D.} = 100 \text{ (S.D./mean)}$$

5. In general, release rates of BP are similar to those of DMBA, except that at the 100-μg level the *in vivo* release of DMBA is slightly slower than that of BP (*cf.* Charts 1*A* and 2*A*). Also, with DMBA a pronounced pellet-to-pellet variability exists in the *in vivo* test at both concentrations, whereas with BP it occurs only at the high concentration and not at the low concentration.

6. Pellets containing a high concentration of BP implanted s.c. show a slow release rate with high pellet-to-pellet variability similar to that of BP pellets inserted into tracheal grafts (*cf.* Chart 1*B* and 1*C*). These *in vitro* and *in vivo* release studies reveal 2 significant deficiencies of the beeswax pellets as delivery devices for the 2 PCH's, BP and DMBA: release rates are too rapid to effect protracted dosing unless high concentrations of PCH's are used, and the release, particularly at high concentrations, shows great pellet-to-pellet variability, which must be due to the conditions (and changing conditions) in the transplant since it does not occur *in vitro*.

Retardation of PCH Release Rate by Modification of Pellet Matrix. The studies presented previously prompted us to explore different possibilities for altering and reducing PCH release. We first explored the effect of adding cholesterol to beeswax in various proportions. In the *in vitro*

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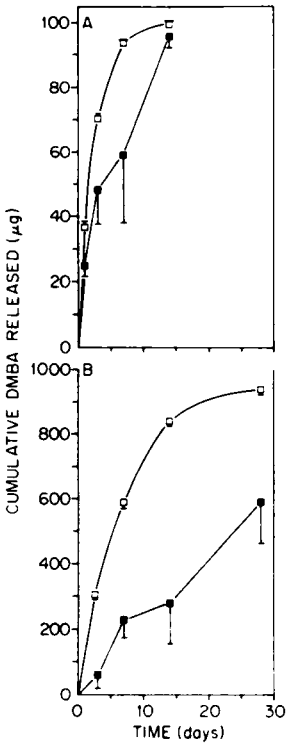

Chart 2. Cumulative release of DMBA from beeswax pellets. *A*, pellets containing 100 μg DMBA. □, *in vitro* release; ■, *in vivo* release in tracheal grafts. Each *point* represents the mean observation from 4 pellets *in vitro* ± S.D. and 6 pellets *in vivo* ± S.D. *B*, pellets containing 1000 μg DMBA. □, *in vitro* release; ■, *in vivo* release in tracheal grafts. Each *point* represents the mean observation from 3 pellets *in vitro* ± S.D. and 6 pellets *in vivo* ± S.D.

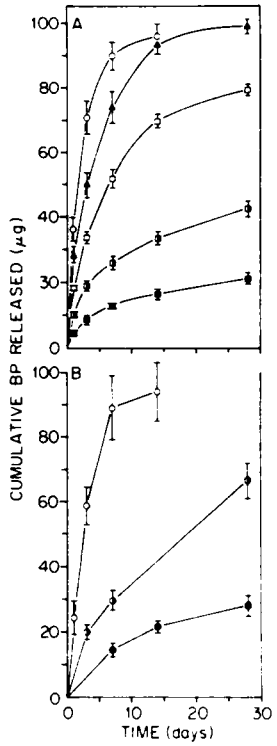

Chart 3. Cumulative release of BP from pellets with modified matrix, at 100 μg BP per pellet. *A*, *in vitro* studies. ○, beeswax pellets; ▲, beeswax:cholesteryl laurate pellets at a ratio of 1:9; □, beeswax:cholesterol pellets at a ratio of 1:1; ◨, beeswax:cholesterol pellets at a ratio of 1:3; ■, beeswax:cholesterol pellets at a ratio of 1:9. Each *point* represents the mean of 2 to 5 observations ± S.D. *B*, *in vivo* studies in tracheal grafts. ○, beeswax pellets; ◑, beeswax:cholesterol pellets at a ratio of 1:3; ●, beeswax:cholesterol pellets at a ratio of 1:9. Each *point* represents the mean of 4 to 6 observations ± S.D.

studies, cholesterol and cholesteryl laurate were used. As illustrated by the data summarized in Chart 3*A*, cholesteryl laurate only marginally reduced the *in vitro* release rate of BP, even when used at a ratio of 1:9 (beeswax:laurate). Cholesterol was much more effective in this regard at proportions ranging from 1:1 to 1:9 (beeswax:cholesterol). At the latter concentration the average amount of BP released *in vitro*, within 1 week, from pellets containing 100 μg BP was ~13% compared to ~90% released during the same time from pure beeswax pellets.

A similar retardation of BP release from pellets was observed in the *in vivo* studies (Chart 3*B*). With a beeswax:cholesterol ratio of 1:3, only 30% of BP was released within 1 week, compared to 90% from beeswax pellets; at a ratio of 1:9, the release was further reduced to 15% during the first week. Also significantly reduced was the pellet-to-pellet variation. For instance, in the case of *in vivo* release from 100-μg-BP beeswax pellets, the percentage of S.D. ranged from 23 to 45 at different time points (Chart 1*A*), whereas, in the case of *in vivo* release from 100 μg BP in the 1:9 beeswax:cholesterol pellets, the percentage of S.D. only ranged from 9 to 14 (Chart 3*B*).

The modification of the pellets by a cholesterol matrix mixed with small amounts of beeswax provides a delivery system for the 2 PCH's with markedly reduced and controllable release rates, with the rate of release depending on the cholesterol concentration. Also markedly reduced is the pellet-to-pellet variability of release.

BP Release from Carbon Particles Incorporated Into Beeswax Pellets. Previous studies in our laboratory had shown that BP adsorbed on activated charcoal particles is slowly released when such particles are introduced into the lungs of rodents (1). We decided to test whether this approach could be of use in the tracheal transplant system.

BP was adsorbed on charcoal particles in BP:carbon ratios of 1:1, 1:3, and 1:9. The BP:carbon was then incorporated into beeswax pellets, and the release of BP from the pellets was studied *in vitro*. As shown in Chart 4*A*, at the BP:carbon ratios of 1:3 and 1:9, the release rates are markedly reduced, compared to the release of BP from beeswax pellets. At the highest carbon concentration, <40% of the BP was released within 4 weeks.

Pilot studies carried out with transplanted hamster, instead of rat, tracheas showed that the same effect was obtained *in vivo* (not shown). The pellets used for these studies were only one-third of the weight of the pellets used for rat tracheas, but they contained 250 μg BP per pellet. At the BP:carbon ratio of 1:9, ~20% of the BP was released within 4 weeks.

Release of TPA from Beeswax Pellets. The tracheal transplant system is potentially very useful for study of the effects of cocarcinogens and promotors or of chemicals that are suspected to have such activities in respiratory carcinogenesis. For performance of such studies, it is essential to have a system of delivery for these substances, which allows exposure of the epithelium for extended periods of time and at dose rates compatible with survival of the target tissues. To determine whether beeswax would

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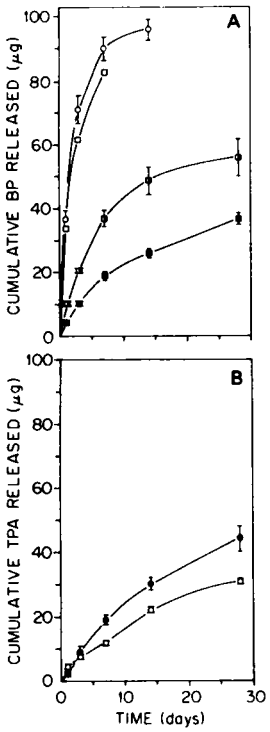

Chart 4. *A*, *in vitro* release of BP from carbon:beeswax matrix. BP was adsorbed to charcoal particles in varying ratios, at 100 μg BP per pellet. ○, BP in beeswax; □, BP:carbon at a ratio of 1:1 in beeswax; ◨, BP:carbon at a ratio of 1:3 in beeswax; ■, BP:carbon at a ratio of 1:9 in beeswax. Each *point* represents the mean observation from 2 to 4 pellets ± S.D. *B*, cumulative release of TPA from beeswax pellets, at 100 μg TPA per pellet. ○, *in vitro* release; ●, *in vivo* release in tracheal grafts. Each *point* represents the mean observation from 4 pellets *in vitro* ± S.D. and 6 pellets *in vivo* ± S.D.

be a feasible matrix to make TPA-containing pellets, we studied *in vitro* and *in vivo* release with a TPA concentration of 0.41% (w/w; 100 μg TPA per pellet). The results of this experiment are summarized in Chart 4*B*. The amount of TPA released per pellet within 4 weeks was 31% *in vitro* and 45% *in vivo*. The percentages of S.D. ranged from 3 to 11 *in vitro* and 7 to 25 *in vivo* for different time points. Thus, the release of TPA from beeswax is much slower than that of the 2 PCH's, BP and DMBA, and the *in vivo* variability of release is also much less. It appears that, with a starting concentration of 0.4% TPA, the transplants could be exposed to this promotor for at least 2 to 3 months.

Histological Observations. We have on various occasions (2–4, 6, 10) given detailed accounts of the histology of normal tracheal grafts and tracheal grafts containing beeswax pellets, and we have described the reaction of the tracheal epithelium to the 2 PCH's, BP and DMBA. We can therefore be brief in this report and describe only those morphological features that might have relevance to the release of the PCH's. The epithelia of untreated established tracheal transplants were, morphologically, virtually indistinguishable from those of normal, *in situ* tracheas. None of the compounds used to make the pellet matrix produced marked changes of the tracheal mucosa. Beeswax pellets caused mild and short-lived hyperplasia; the effect of cholesterol:beeswax pellets was not noticeably different. Beeswax pellets containing carbon particles, however, caused somewhat stronger reactions, resulting in appearance of hyperplastic and transitional epithelium (*i.e.*, epithelium resembling the transitional epithelium of the bladder). Also seen were foci of flattened atrophic epithelium.

The inclusion of carcinogens BP and DMBA in the pellets brought about far more severe changes in the transplants. During the first 3 days, DMBA caused a basal cell hyperplasia, and by 2 weeks the normal lining was replaced by a uniform keratinizing squamous epithelium accompanied by marked submucosal edema. With BP, metaplasia was only rarely seen; instead, marked hyperplasia and thick transitional epithelium appeared during the first few days and weeks. The high doses of the 2 PCH's also caused marked changes of the submucosa, resulting in connective tissue condensation and hyalinization at 2 to 4 weeks.

TPA elicited the most severe response. During the first 3 days of exposure, there was a marked granulocytic infiltration accompanied by epithelial hyperplasias as well as focal necrosis. By 7 days the transplants were characterized by marked inflammatory changes extending to the paratracheal connective tissue. Severe edema and granulocytic as well as mononuclear infiltrates were widespread, and an increase of fibroblastic elements was noticeable. By 28 days the severe reaction regressed, although some tracheas still showed a moderate degree of inflammation. Hyperplasia was still evident in most tracheas.

DISCUSSION

Previous studies have demonstrated the usefulness of the tracheal transplant model as a tool in experimental lung cancer research (for review see Ref. 9). The attractiveness of this new approach is also apparent since several other laboratories have adopted it with only minor modifications (7, 8). However, our work during the past 2 years with this experimental system has revealed several shortcomings, most of them related to the carcinogen delivery system used to bring the test substance in direct contact with the tracheal epithelium.

The studies described here have pinpointed some of the problems inherent in the "drug release system" chosen by us for the protracted delivery of carcinogens and have also pointed the way to elimination of these deficiencies or at least to a reduction of them to an acceptable level. The main problem we were confronted with was the great variability of release rates from transplant to transplant. Our studies indicate that this is not due to variability inherent in the PCH-containing pellets, since we found that the *in vitro* release characteristics of such pellets are very reproducible with only minor pellet-to-pellet variation. This was found to be true for BP and DMBA at concentrations of either 0.41 or 4.10% for either compound. Thus, it is clear that the variation is brought about by the influence of the tracheal tissue and its secretions (and possibly by the influence of the host animal) on the release of the carcinogen. The variation appears to be associated with the toxicity of the released material and the resulting tissue modifications. At high doses of BP (pellets containing 4.1%) and at low as well as high doses of DMBA (pellets containing 0.41 or 4.1%), large variations are encountered although not to the same extent as with low doses of BP (0.41%). This corresponds to the known toxicity of these 2 PCH's (DMBA is the

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more toxic of the 2) and the actual morphological changes observed after implantation of pellets into transplanted tracheas. Both of the DMBA concentrations as well as the high BP concentration cause massive hyperplastic (metaplastic) reactions of the epithelium, severe edema, and inflammation of submucosa and paratracheal connective tissue. In contrast, only mild pathological tissue changes are observed following insertion of low-concentration BP pellets. The massive tissue alterations following exposure to DMBA and high doses of BP are probably associated with changes in fluid exchange in these tissues (alteration of secretion, tissue permeability, and circulation; edema and exudation; thickening of epithelium and connective tissue layers). It seems reasonable to assume that such changes in turn affect the release of the test substance from the pellets. This notion is supported by the finding that, 4 weeks after implantation, s.c. transplanted pellets containing 4.1% BP virtually stopped releasing the PCH. At this time the pellets were firmly encapsulated by dense connective tissue. Thus, the released test substance, if highly toxic, causes tissue changes that in turn affect the further release of that substance.

Our studies strongly indicate that the early rapid release of PCH's from the beeswax matrix is the major fault of the system, bringing about or markedly accentuating all other problems. Since toxicity is associated with all of the substances with which we are concerned, it is essential that it be minimized. This should be possible by reducing release rates. The experiments with modified pellet matrices indeed bear out that this is an effective strategy. The rate of release of BP from pellets containing cholesterol:beeswax mixtures of varying proportions was progressively reduced with increasing cholesterol concentration, reducing at the same time the pellet-to-pellet variability of release. We interpret this as an indication that lower dose rates cause less dramatic, toxic tissue changes, resulting in lessened influence of such changes on release and less variability. With these experiments we have accomplished a dual purpose. We have found a way to control the rate of PCH release and effect slower release rates and thus have obtained more protracted exposures, and at the same time we have reduced the variability in release rates encountered previously because of the marked tissue changes caused by the rapid release of PCH's.

An alternative to cholesterol is the adsorption of PCH to charcoal and the incorporation of the BP:charcoal mixture into the beeswax matrix. The *in vitro* studies suggest that this also significantly reduces the release rate of the PCH. However, this method appears to have several disadvantages. First, it is not easy to produce a stable particle suspension in liquid beeswax. This makes it difficult to produce pellets with consistent amounts of PCH. Second, an *in vivo* study performed with charcoal incorporated into beeswax suggested that the charcoal itself caused some toxic changes of the tracheal epithelium. A similar study with cholesterol:beeswax pellets showed no tissue reaction beyond that seen normally with beeswax pellets. Thus, it seems that for the moment the cholesterol:beeswax mixtures provide the more desirable means to achieve a sustained, slow release of PCH from i.t. pellets and to control release rate by varying cholesterol concentration. We are continuing to explore alternatives to this approach and are examining various synthetic polymers for encasing or coating carcinogen pellets and for regulating release rates by varying membrane thickness. Another possibility for effecting slow release was recently described by Mossman and Craighead (8). These investigators adsorbed the PCH to Lycra fibers of standard length and thickness. When such fibers are placed into transplanted hamster tracheas, continuous release of the PCH results, lasting for many weeks.

Finally, by using beeswax pellets we explored for the first time methods for exposing tracheal transplants continuously to phorbol ester TPA. The *in vivo* study showed a release rate of approximately 1.1 μg TPA per day for a period of 28 days. The experiment was terminated at that time. TPA release from beeswax thus is considerably slower than PCH release. Surprisingly little pellet-to-pellet variation was observed. The massive tissue reaction observed (particularly between Days 8 and 14) suggests, however, that this rate of TPA delivery to the tracheal tissue may still be somewhat too rapid. Further studies will be initiated to clarify this point. The development of an *in vivo* initiation-promotion model with respiratory tract epithelium would be an important advancement in respiratory carcinogenesis studies.

Through the experiments reported here, a major deficiency of the tracheal transplant model was corrected by obtaining better control over continuous carcinogen delivery to the target tissue and by reducing variability in dose rates. With this improvement the experimental system should considerably gain in its usefulness for studies of neoplastic development in respiratory tract tissues.

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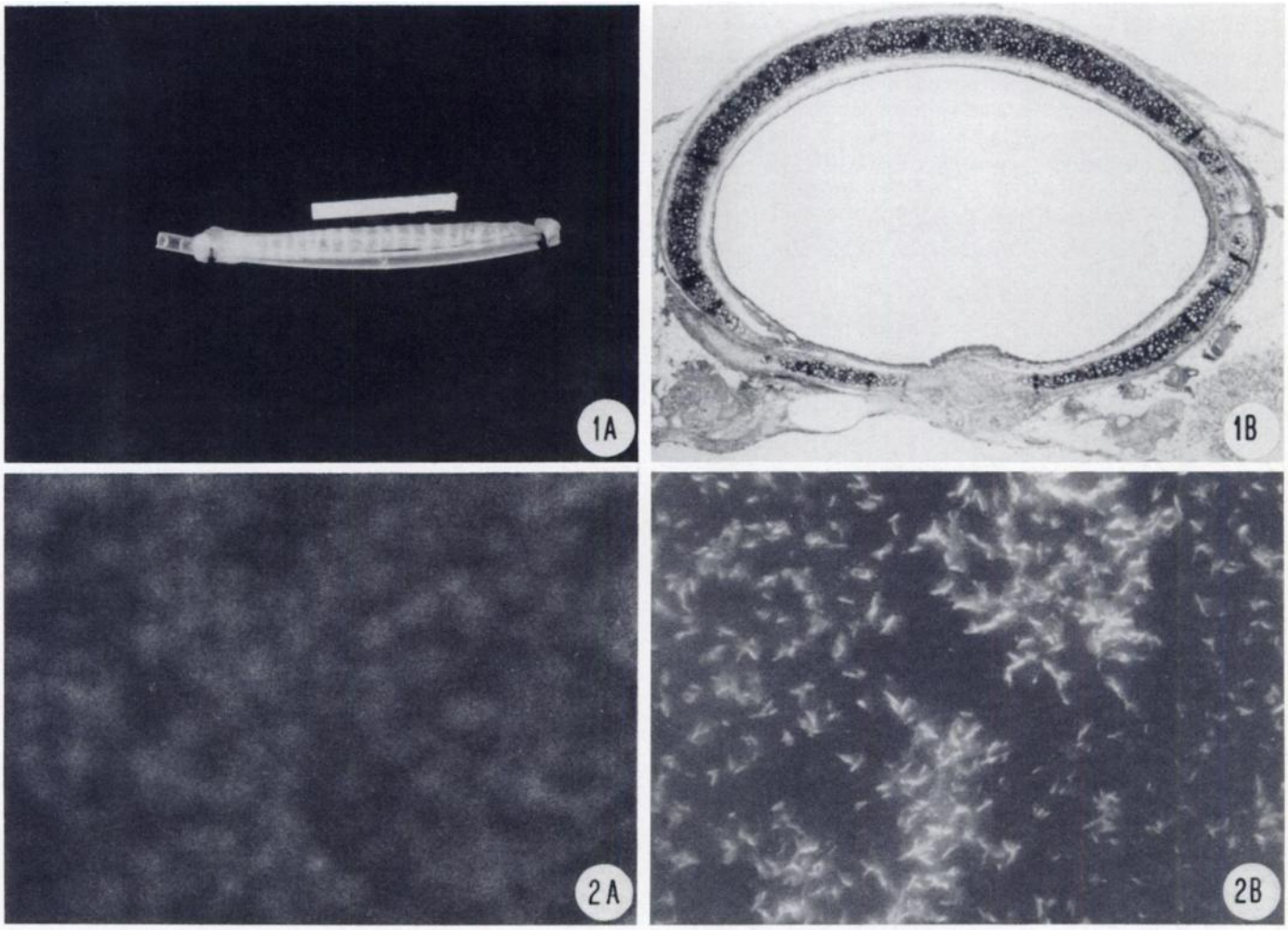

Fig. 1. *A*, rat trachea sutured to polyethylene tubing. These were implanted s.c. for tracheal transplant studies. Next to it is a beeswax pellet of the type placed in the lumen of established transplants for determining *in vivo* release characteristics. × 1.5. *B*, cross-section of an established tracheal transplant showing normal epithelial morphology. H & E, × 32.

Fig. 2. *A*, photomicrograph of a 5-μm-thick section of BP:beeswax matrix [0.40% (w/w) BP] taken with a reflecting microscope and UV and showing absence of any crystalline undissolved BP. × 400. *B*, photomicrograph of a 5-μm-thick section of BP:beeswax matrix [4.0% (w/w) BP] showing the presence of undissolved BP crystals. × 400.

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