A Survey of Compounds for Activity in the Suppression of Mouse Sebaceous Glands*

FRED G. BOCK AND RHODA MUND

(Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo 3, N.Y.)

Sebaceous gland tests have been used for assay of the carcinogenic potency of cigarette smoke condensates (24, 26). Such assays can be completed in a short time with a moderate expenditure of animals and material. If reliable, the assays would greatly reduce the time required for the fractionation of crude materials causing skin tumors in mice by pinpointing effective fractions. Another value of the assay might be its use to monitor levels of carcinogens in various industrial operations and in commercial products.

Theoretical interest in the relationship between sebaceous glands and carcinogenesis has been stimulated by a number of reports (3, 16, 17, 21–23). Simpson and Cramer (23) suggest that sebaceous glands may protect the skin from methylcholanthrene. On the other hand, it is well known that for lipides the sebaceous glands serve as a route of penetration through the skin. Lacassagne and Latarjet (12) observed that the pilosebaceous apparatus appeared important for the development of skin tumors induced by ultraviolet irradiation followed by methylcholanthrene painting.

This investigation was undertaken to learn more about the nature of sebaceous gland suppression through a study of the activity of a variety of compounds.

MATERIALS AND METHODS

Swiss mice, 55–65 days of age, were used for the assay. The hair from approximately two-thirds of the back was shaved off with an Oster electric clipper (size 0000 head). On the following 3 days, each animal was treated, twice daily, with 0.2 ml. of test solution by allowing the liquid to flow from a pipette onto the center of the clipped area. The mice were sacrificed 1 week after being clipped (4 days after the last application), and the treated skin was pinned, fur down, on a dissecting board. Vigorous scraping with the dull edge of a Bard-Parker #12 blade removed the subcutaneous fat. Following scraping, the skins were suspended from a cork by means of four pins. They were placed in physiological saline for a period of 3–6 hours, then immersed in 10 per cent formalin overnight.

* This investigation was supported in part by a research grant from the Tobacco Industry Research Committee.

Received for publication February 26, 1958.

The staining procedure was described in an earlier publication (2). Whole mounts of mouse skin were stained; thus, the number of glands did not depend upon the orientation of the sections. Where possible, benzene was used as a solvent, while ethanol or acetone was used for benzene-insoluble materials. The minimum quantity that removed at least 50 per cent of the sebaceous glands from the treated area of one animal was defined as one unit of activity. For pure compounds, the sebaceous gland suppression index was defined as the number of units of activity per gram.

Concentrations causing destruction of the entire epidermis should never be employed to avoid confusion between specific sebaceous gland suppression and general caustic effects.

Because the sebaceous gland test seemed to give inconsistent results, long-range carcinogenic tests were conducted with 6-methylbenzo[*a*]pyrene and 7,9-dimethylbenz[*c*]acridine. In the former case, 0.25 ml. of a 0.4 per cent solution of the hydrocarbon in acetone-benzene (2:1) was applied to the shaved backs of 40 female Swiss mice twice weekly for 23 weeks. In the latter case, 0.25 ml. of a 0.3 per cent solution of 7,9-dimethylbenz[*c*]acridine in acetone was dropped on the backs of twenty female Swiss mice twice weekly for 24 weeks. 3-Methylcholanthrene at the same dosage level and in the same solvent was used for comparison in each series.

RESULTS

Table 1 lists the sebaceous gland suppression index of compounds that have been tested. Serial dilution permits semiquantitative evaluation of the suppression index of various substances. As described in our earlier publication, the assays are sensitive enough to demonstrate twofold differences in concentration of pure compounds.

The following fused-ring hydrocarbons were inactive when tested in benzene at the maximum concentrations listed (in percentages): perylene, 0.33; naphthacene, 0.3; benzo[*e*]pyrene, 1; 11-benzo[*b*]fluorene, 1.6; phenanthrene, 10; pyrene, 20; anthracene, 1.7; naphthalene, 25; 5-benzo[*b*]carbazole, 0.2; 1,9-benzanthrone, 1.0; acenaphthalene, 30; tetrahydroacenaphthalene, 100; 9-methylanthracene, 5; difluorenyl, 2; rubrene, 0.9; fluorene, 10; fluoranthene, 1; and benzo[*g,h,i*]perylene, 1. Four carcinogens active at sites other than the skin were tested and found not to destroy the sebaceous glands. These were (percentages): 2-aminoanthracene, 1.5; *p*-dimethylaminoazobenzene, 18; 2-acetylaminofluorene, 1.8; 2-naphthyl-

TABLE 1

SEBACEOUS GLAND SUPPRESSION INDEX OF VARIOUS COMPOUNDS

COMPOUND	MAXIMUM CONCENTRATION OF INACTIVE COMPOUNDS TESTED (per cent)	SKIN CARCINOGENIC POTENCY* (5, 9, 10, 18, 19)	SUPPRESSION INDEX
Benz[*a*]anthracene derivatives			
7,12-Dimethylbenz[*a*]anthracene		A	10,000
6-Methylbenzo[*a*]pyrene		A	10,000
3-Methylcholanthrene		A	10,000
Benzo[*a*]pyrene		A	3,000
Dibenz[*a,h*]anthracene		A	3,000
7-Methylbenz[*a*]anthracene		A	2,000
Benz[*a*]anthracene		D	1,000
5-Methylbenz[*a*]anthracene		D	1,000
6-Methylbenz[*a*]anthracene		D	1,000
8-Methylbenz[*a*]anthracene		W	1,000
9-Methylbenz[*a*]anthracene		W	1,000
11-Methylbenz[*a*]anthracene		D	1,000
2-Methylbenz[*a*]anthracene		O	300
12-Methylbenz[*a*]anthracene		W	300
1-Methylbenz[*a*]anthracene		O	300
3-Methylbenz[*a*]anthracene		O	100
7-Propylbenz[*a*]anthracene			100
4-Methylbenz[*a*]anthracene	1.0	W	0
10-Methylbenz[*a*]anthracene	1.0	D	0
Benzo[*c*]phenanthrene derivatives			
2-Methylbenzo[*c*]phenanthrene		D	65
5-Methylbenzo[*c*]phenanthrene		A	65
6-Methylbenzo[*c*]phenanthrene		A	65
3-Methylbenzo[*c*]phenanthrene	1.5	W	0
4-Methylbenzo[*c*]phenanthrene	1.5	W	0
Other compounds active against skin			
7,9-Dimethylbenz[*c*]acridine		A	300
1,3-Bis[*2,3*-epoxy propoxy]benzene		W†	100
Beta-propiolactone		W	30
Butadiene diepoxide		W†	30
Cyclovinyl diepoxide	10	W†	0
Di[*2,3*-epoxypropyl]ether	1	W†	0
Epoxy soya oil	30	W†	0
Quinone	10	W	0
1,4-Naphthoquinone	0.5	W	0
Urethan	10	I	0
Croton oil	10	P	0
Phenol	1	P	0
Iodoacetic acid	0.5	P	0
Dodecylbenzene	100	B	0
Oleic acid	100	H	0
Squalene	100	H	0
Miscellaneous sebaceous gland suppressors			
Colchicine		O	1,000
Chrysene		D	200
Triphenylene		O	50
9-Bromophenanthrene			30
Lauroyl peroxide		O†	<30

* Explanation of symbols:
A: Strongly active compound.
W: Weakly active compound.
D: Compound with doubtful or marginal activity.
O: Inactive compound.
I: Initiator.
P: Promoter.
B: Potentiator of the activity of other carcinogens.
H: Compound producing hyperplasia.

† Paul Kotin, personal communication

amine, 1. The following miscellaneous compounds were inactive against sebaceous glands: phenanthrenequinone, 100; octene, 100; 1,2-naphthoquinone, 1; hydroquinone, 0.5; allyl propionate, 10; coumarin, 10; *p*-bromophenol, 1; *o*-iodophenol, 10; 2,4-dinitrophenol, 10; benzene, 100; acetone, 100; *n*-heptane, 100; pet. ether, 30–60, 100; pet. ether, 62–69, 100; toluene, 100; ethanol, 100; N,N-dimethylformamide, 100; trioctanoin, 100; hydrocortisone, 1; cycloöctatetraene, 30; azulene, 2; ascorbic acid, 2.5; deoxycholic acid, 2.5; allyl thiourea, 2.5; crotonic acid, 5; phenyl acetate, 10; anisole, 10; anthraquinone, 0.5; tetrahydronaphthalene, 100; 2-naphthol, 10; *p*-fluorophenol,

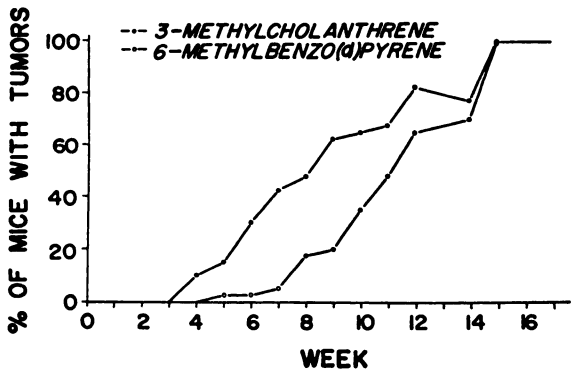

CHART 1.—Induction of skin tumors with 6-methylbenzo[*a*]pyrene. The tumors include both papillomas and carcinomas. Semiweekly applications of 0.4 per cent benzene-acetone solutions were used. No tumors appeared in control animals treated with solvent alone.

3; linseed oil, 100; linoleic acid, 100; allyl urea, 10; styrene oxide, 10; cumene hydroperoxide, 10.

Reproducibility of results was good. During a 3-year period, three different analysts obtained comparable results with a number of active compounds. Choice of solvent affected the results when methylcholanthrene was used. Benzene, acetone, and ethanol appeared to be interchangeable. On the other hand, a 0.06 per cent solution of methylcholanthrene in heavy mineral oil caused the disappearance of less than 20 per cent of the glands. This effect was less than that caused by a 0.006 per cent solution in benzene.

The tumorigenic potency of 6-methylbenzo[*a*]pyrene is demonstrated in Chart 1. The tumors referred to in the chart include both papillomas and carcinomas, with a majority of papillomas. Loss of hair during the resting period of hair growth cycle was observed. These observations extend an earlier report (20) that 6-methylbenzo[*a*]pyrene is a strong carcinogen when injected subcutaneously.

Comparison of 7,9-dimethylbenz[*c*]acridine with 3-methylcholanthrene is illustrated in Chart 2. The high carcinogenic potency of the former compound has been described by Lacassagne *et al.* (13). Our results established that the commercial product we employed behaved as expected in our particular strain of mice.

DISCUSSION

High levels of sebaceous gland suppressor activity are clearly associated with the benzanthracene structure. Within this class of compounds the suppression index is roughly parallel to mouse skin carcinogenic potency. A comparison of 7-methylbenz[*a*]anthracene with 7-propylbenz[*a*]anthracene is of particular interest, since lengthening the side chain strongly reduces sebaceous gland suppressor activity just as it does carcinogenic activity(20).

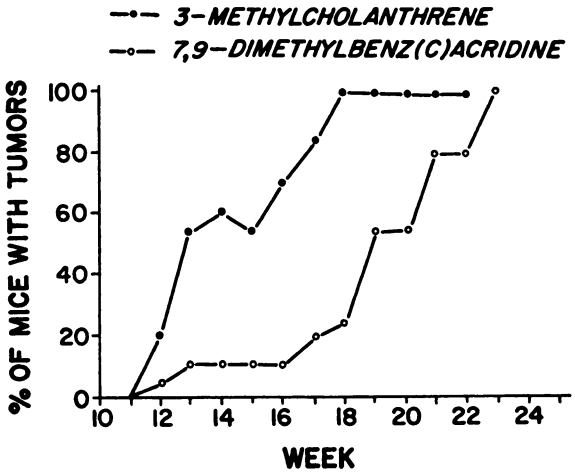

CHART 2.—Induction of skin tumors with 7,9-dimethylbenz[*c*]acridine. Semiweekly applications of 0.3 per cent acetone solutions were used. No tumors appeared in control animals treated with solvent alone.

Mouse skin carcinogens in which the benzanthracene structure is absent do not destroy sebaceous glands. This is most striking in the case of 7,9-dimethylbenz[*c*]acridine which, for this mouse stock, is nearly as tumorigenic as methylcholanthrene, while its suppression index is only 3 per cent as great. Similar evidence is afforded by the methylbenzo[*c*]phenanthrenes which have produced skin tumors in mice (1). A variety of nonaromatic mouse skin carcinogens is also inactive against sebaceous glands.

On the other hand, colchicine has sebaceous gland suppressor activity comparable to carcinogenic benzanthracene derivatives. Thus, sebaceous gland suppression is neither necessary nor sufficient as a criterion of carcinogenic activity. Harde

(8) reported production of multiple papillomas in three of 26 mice treated with a colchicine pomade. In contrast, Hamperl (7) failed to observe skin tumors after painting mice for 5 months. We have painted the backs of Swiss mice with various concentrations of colchicine in benzene for periods up to 16 months. No skin tumors have been observed, although the treatment produced epilation and occasional ulceration.

In spite of these discrepancies, sebaceous gland tests can be of practical value. Serial dilution gives a typical dose-response curve (2) and thus permits semiquantitative estimation of activity. The procedure requires short-term exposure of the animals to experimental conditions so that the influence of uncontrolled environmental factors is greatly reduced. An important example of such factors is the hair growth cycle, which can be controlled in these short-term assays. It should be reemphasized that care must be exercised in the interpretation of results, unless chemical evidence demonstrates that the test materials are benzanthracene derivatives.

Sebaceous gland assays may be used to determine the efficiency of isolation of suspected carcinogens from crude materials. In such cases a semiquantitative estimate of yield and activity per gram can be obtained. This type of application has been made by Suntzeff *et al.* (25). We have found it to be of great value in our laboratory as well.

A second application of such tests lies in determination of suppressor activity per unit of weight and per unit of chromophore at different wavelengths (6). Comparison of these values with those of known compounds can often be used to reject the hypothesis that a particular substance is responsible for a major part of the biologic activity. The technic can be employed for crude materials which have background absorption preventing exact identification. Table 2 presents values for some sebaceous gland suppressors of interest. Partially refined fractions will often have values several times higher than could be accounted for by assuming that all the chromophoric material is a specific compound. Background absorption generally limits the use of this technic to the higher wave-lengths. An example of this application is demonstrated by the case of two fractions derived from cigarette smoke condensate. A fraction 1 contained 48 units of suppressor activity and, when diluted 2500 times, had an optical density of 0.6 at 360 mμ. The $\frac{\text{suppressor units/ml}}{\text{optical density}} \times 100$ equaled 3, well within the range of values from Table 2. On the other hand, fraction 4 contained approximate 400 units of suppressor activity and, when diluted 1000 to 1, had an optical density of .75. Accordingly, the $\frac{\text{suppressor units/ml}}{\text{optical density}} \times 100$ equaled 53. It is clear that neither benzo[*a*]pyrene nor benz[*a*]anthracene could have accounted for all the suppressor activity of fraction 4.

Another example of how sebaceous gland tests are of value is demonstrated in the case of 6-methylbenzo[*a*]pyrene. The compound was sold as the 5-methyl derivative—reportedly a weak carcinogen (4). However, its sebaceous gland suppression index was equal to that of 3-methylcholanthrene and 7,12-dimethylbenz[*a*]anthracene, thus suggesting a very high carcinogenic potency. The long-term skin painting experiment substantiated this belief, and subsequently a communication from the manufacturer confirmed our presumption that the compound was incorrectly labeled.

If the sebaceous glands have a defensive function, their apparent effect is limited to cases involving benzanthracene derivatives. The relative stability of the benzacridines (11) may be a factor to consider in this respect. However, there does not seem to be any established relationship between chemical activity and sebaceous gland-suppressor activity.

Advantages of sebaceous gland studies for interpretation of the mechanism of skin carcinogenesis should not be overlooked. For the benzanthracene compounds, pathways leading to sebaceous gland suppression on one hand and carcinogenesis on the other probably have some steps in common. Properties such as permeability, solubility, decomposition, and metabolic degradation are likely to affect both processes in the same way.

TABLE 2

SEBACEOUS GLAND SUPPRESSION AND OPTICAL DENSITY

	$\frac{\text{Suppression units/ml}}{\text{Optical density}} \times 100$				
	360 mμ	383 mμ	395 mμ	403 mμ	408 mμ
6-Methylbenzo[*a*]pyrene	22	14	10	84	48
Benzo[*a*]pyrene	4	3	59	19	76
Dibenz[*a*,*h*]anthracene	64	210	55	>830	>830
Benz[*a*]anthracene	7	29	>230	>230	>230

Thus, the parallel between the activities of 7-methyl and 7-propyl benz[*a*]anthracene would indicate that the chain length affects an early common stage. In a similar way, one might expect that the low carcinogenicity of dibenz[*a*,*h*]anthracene in mineral oil (27) is due to poor absorption of the compound from this solvent. The sebaceous gland data tend to substantiate this supposition.

In considering the various points listed above, it is necessary to discuss the technic of the assay. Several factors must be carefully controlled in making the analyses. First of all, each new compound should be surveyed over a broad range of concentrations. Many compounds will destroy the entire epidermis when applied in high concentration. It seems reasonable that such general caustic effects should not be included as specific sebaceous gland suppression. Thus, less concentrated solutions should be employed. Other compounds may cause less sebaceous gland suppression from a concentrated solution than they do from a dilute one. While we have not observed this property in highly active compounds, it was true for β-propiolactone, which was more active at the 3 per cent than at the 10 per cent level.

Secondly, the state of the mouse should be discussed. Montagna and Chase (16) have noted that, if hair follicles are active, new sebaceous glands are formed 5 days after application of methylcholanthrene. Suntzeff *et al.* (25) have reported lack of uniformity in gland destruction in areas of mouse skin with growing hair. Obviously, reliable data can best be collected during the telogen phase of hair development.

The method of tissue preparation is important. Because of the anatomic position of the glands, variable numbers of them will appear in sections prepared in the usual manner. Accordingly, a procedure that permits examination of an entire area of skin is essential for even semiquantitative counts of the glands. The method developed by Suntzeff meets this criterion. The procedure that we employ also satisfies this requirement but is limited by its dependence upon the characteristics of the stain. The nature of sebaceous glands has been reviewed by Montagna (14, 15). In the light of his cytochemical studies, it seems possible that small remnants of glands consisting wholly of peripheral cells might not be stained by the Nile Blue technic. This factor should not affect data within the same laboratory but might interfere with comparisons between groups using different staining procedures. It should be pointed out, however, that the agreement between the data reported above and those of Suntzeff is very good, even though different dose schedules were employed.

The timing and dose schedules used by us are largely arbitrary. They have been designed to permit the maximum response from cigarette smoke condensate. In this case nicotine toxicity and low levels of suppressor activity required frequent painting. For pure compounds, a single application would be more convenient, but would give a different quantitative result. It would seem reasonable to vary the dose schedule according to the needs of an individual experiment, taking into consideration the solubility, toxicity, stability, and times of response to the particular test material.

SUMMARY

We have measured the sebaceous gland suppression index of 103 compounds. Thirteen of these had an index of 1,000 or more, while 74 had no measurable activity. High levels of sebaceous gland-suppressor activity were clearly associated with the benz[*a*]anthracene structure. Within this class of compounds the suppression index was roughly parallel to mouse skin carcinogenic potency.

Sebaceous gland suppression was neither necessary nor sufficient as a criterion of skin carcinogenic activity. Thus, the potent carcinogen 7,9-dimethylbenz[*c*]acridine had very weak suppressor activity, while colchicine was moderately active though not a skin carcinogen.

Subject to these limitations, sebaceous gland tests can be of great value for the study of carcinogenesis. They can be employed to suggest the presence or absence of benz[*a*]anthracene-type carcinogens and to measure efficiency of their isolation. To a lesser extent, the tests are helpful in identification of partially purified hydrocarbons. Uses of the test to study the mechanism of skin carcinogenesis were also pointed out.

6-Methylbenzo[*a*]pyrene was demonstrated to be a very active skin carcinogen.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Melvin Newman for generous supplies of the series of methylbenz[*a*]anthracenes and methylbenzo[*c*]phenanthrenes. They are indebted to Dr. Paul Kotin for samples of the various epoxides and peroxides. The technical assistance of Mrs. Dolores Mickens is also gratefully acknowledged.

REFERENCES

1. BADGER, G. M.; COOK, J. W.; HEWETT, C. L.; KENNAWAY, E. L.; KENNAWAY, N. M.; MARTIN, R. H.; and ROBINSON, A. M. The Production of Cancer by Pure Hydrocarbons. Proc. Roy. Soc., London, s.B, 129:439–67, 1940.
2. BOCK, F. G., and MUND, R. Evaluation of Substances Causing Loss of Sebaceous Glands from Mouse Skin. J. Invest. Dermatol., 26:479–87, 1956.
3. CAMBEL, P. Fluorescence Studies of Carcinogens in the Rat Skin. Cancer Research, 11:370–75, 1951.
4. DUNLAP, C. E., and WARREN, S. Chemical Configuration and Carcinogenesis. Cancer Research, 1:953–54, 1941.

5. Flesch, P. Hair Loss from Sebum. Arch. Dermatol. & Syph., **67**:1–9, 1953.
6. Friedel, R. A., and Orchin, M. Ultraviolet Spectra of Aromatic Compounds. New York: John Wiley & Sons, 1951.
7. Hamperl, H. Über chronische Colchicinwirkung, Klin. u. Praxis, **1**:186–87, 1946.
8. Harde, E. Occurrence of Tumors in Mice during Prolonged Treatment with Colchicine. 3d Internat. Cancer Cong., 126, 1939.
9. Hartwell, J. L. Survey of Compounds Which Have Been Tested for Carcinogenic Activity. Public Health Service Publication #149. Washington, D.C.: United States Government Printing Office, 1951.
10. Horton, A. W.; Denman, D. T.; and Trosset, R. P. Carcinogenesis of the Skin. II. The Accelerating Properties of Aliphatic and Related Hydrocarbons. Cancer Research, **17**:758–66, 1957.
11. Lacassagne, A.; Buu-Hoï, N. P.; Daudel, R.; and Zajdela, F. The Relation between Carcinogenic Activity and the Physical and Chemical Properties of Angular Benzacridines. Adv. Cancer Research, **4**:315–69, 1956.
12. Lacassagne, A., and Latarjet, R. Action of Methylcholanthrene on Certain Scars of the Skin in Mice. Cancer Research, **6**:183–88, 1946.
13. Lacassagne, A.; Rudali, G.; Buu-Hoï, N. P.; and Lecocq, J. Activité cancérigène de certains dérivés méthylés des benzacridines angulaires. Compt. rend. Soc. de biol., **139**:955–57, 1945.
14. Montagna, W. The Cytology of Mammalian Epidermis and Sebaceous glands. Int. Rev. Cytol., **1**:265–304, 1952.
15. ———. The Structure and Function of Skin. New York: Academic Press, Inc., 1956.
16. Montagna, W., and Chase, H. B. Redifferentiation of Sebaceous Glands in the Mouse after Total Extirpation with Methylcholanthrene. Anat. Rec., **107**:83–92, 1950.
17. Pullinger, B. D. The First Effects on Mouse Skin of Some Polycyclic Hydrocarbons. J. Path. & Bact., **50**:463–71, 1940.
18. Roe, F. J. C., and Glendenning, O. M. The Carcinogenicity of β-Propiolactone for Mouse Skin. Brit. J. Cancer, **10**:357–62, 1956.
19. Rusch, H. P.; Bosch, D.; and Boutwell, R. K. The Influence of Irritants on Mitotic Activity and Tumor Formation in Mouse Epidermis. Acta Unio Internat. contra cancrum, **11**:699–703, 1955.
20. Shear, M. J., and Leiter, J. Studies in Carcinogenesis. XIV. 3-Substituted and 10-Substituted derivatives of 1,2-Benzanthracene. J. Nat. Cancer Inst., **1**:303–36, 1940.
21. Simpson, W. L.; Carruthers, C.; and Cramer, W. Loss of Carcinogenic Activity When Methylcholanthrene Is Dissolved in Anhydrous Lanolin. Cancer Research, **5**:1–4, 1945.
22. Simpson, W. L., and Cramer, W. Fluorescence Studies of Carcinogens in Skin. I. Histological Localization of 20-Methylcholanthrene in Mouse Skin after Single Application. Cancer Research, **3**:362–69, 1943.
23. ———. Sebaceous Glands and Experimental Skin Carcinogenesis in Mice. *Ibid.*, pp. 515–18.
24. Smith, W. E. Sebaceous Gland Activity as a Measure of the Relative Carcinogenicity of Certain Oils and Tars. Bull. New York Acad. Med., s. 2, **32**:77–78, 1956.
25. Suntzeff, V.; Cowdry, E. V.; and Croninger, A. Microscopic Visualization of the Degeneration of Sebaceous Glands Caused by Carcinogens. Cancer Research, **15**:637–40, 1955.
26. Suntzeff, V.; Croninger, A. B.; Wynder, E. L.; Cowdry, E. V.; and Graham, E. A. Use of Sebaceous-Gland Tests of Primary Cigarette Tar Fractions and of Certain Noncarcinogenic Polycyclic Hydrocarbons. Cancer, **10**:250–54, 1957.
27. Twort, J. M., and Twort, C. C. Comparative Activity of Some Carcinogenic Hydrocarbons. Am. J. Cancer, **35**:80–85, 1939.