1 hr, then excess $POCl_3$ was stripped *in vacuo*. A little ice was added and the residue was neutralized with $Na_2CO_3$. Evapn of the $H_2O$, extn of the residue with warm EtOAc, and evapn of the EtOAc gave 6.5 g of a slightly orange oil that crystd on standing. Treatment with charcoal in $Et_2O$ gave, after filtration and evapn of $Et_2O$, 5.8 g (64%) of 38, mp 49–50°. The analytical sample (hexane) had mp 50–51°.

**3,4-Bis(dimethylamino)-1,2,5-thiadiazole (41).** An autoclave was charged with 3,4-dichloro-1,2,5-thiadiazole[13] (15.5 g, 0.10 mole), anhyd $Me_2NH$ (45 g, 1.0 mole), and DMSO (150 ml), then was heated at 120° for 5 hr. The dark mixt was poured into $CHCl_3$ (600 ml), and the soln was washed with several portions of dil NaCl. The NaCl washings were again extd with $CHCl_3$, and the combined $CHCl_3$ portions were again washed with dil NaCl (2 × 500 ml) and with $H_2O$ (2 × 500 ml). Distn of the org phase yielded 8.5 g (49%) of 41, bp 110–112° (20 mm).

**3,4-Bis(dimethylamino)-1,2,5-oxadiazole 2-Oxide (40).** A soln of dichloroglyoxime diacetate[2b] (68.4 g) in $CH_2Cl_2$ (1 l) was added dropwise to a cold (0–5°) soln of anhyd $Me_2NH$ (90 g) in $CH_2Cl_2$ (600 ml). After the addn the soln was refluxed 1 hr, then the solvent was stripped and the residue was extd with 3 portions of 2:1 $Et_2O$–hexane to remove dimethylacetamide. The residue was then extd with several portions of hot EtOAc, and finally with 1:1 THF–EtOH; the combined exts were filtered and evapd, and the resulting solid was made slightly alk with $Na_2CO_3$. $H_2O$ was stripped and the residue was again extd with hot EtOAc to give 33.5 g of crude bis(dimethylamino)glyoxime, mp 125–145°. Recrystn from EtOAc–hexane gave a first crop of 20.8 g (mp 163–165°, 42%) and a second crop of 9 g (mp 166–169°, 18%). The analytical sample (EtOAc–hexane) had mp 169–170°. *Anal.* C, H, N.

A soln of bis(dimethylamino)glyoxime (14.6 g) in 0.5 *N* NaOH (150 ml) at 0–5° was treated dropwise with 5.25% NaOCl (145 g). A white solid separated that was collected by filtration and washed with cold $H_2O$; the yield of 3,4-bis(dimethylamino)-1,2,5-oxadiazole 2-oxide (40) was 11.3 g (79%, mp 45–46°). A second crop (0.9 g, 6%) was recrystd 3 times from pentane, mp 46–48°.

**3,4-Bis(dimethylamino)-1,2,5-oxadiazole (39).** A soln of 40 (0.38 g) in $CHCl_3$ (5 ml) and $PCl_3$ (0.5 ml) was refluxed 1 hr. The red soln was poured onto ice and neutralized with $Na_2CO_3$, then the aq phase was extd thoroughly with $CHCl_3$. The dried $CHCl_3$ exts were evapd to give 0.26 g (75%) of 39 as an oil that solidified on cooling. Recrystn from MeOH–$H_2O$, then from pentane at −20°, gave pure 39, mp 51.5–52°,

**5-(Dimethylamino)-2,3-dihydro-3-methyl-4*H*-1,3,5-thiadiazine-4-thione (54).** A mixt of 1,1,5-trimethyl-2,4-dithiobiuret[1] (1.0 g) and aqueous $H_2CO$ (1 g, 40%) in $H_2O$ (7 ml) was refluxed 1 hr then was stirred overnight at room temp. A cryst white solid and a sticky yellow solid separated from soln; the white solid (0.22 g, mp 167–171°) was separated mechanically and recrystd from MeOH to give pure 54, mp 169–171°.

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# Molecular Weight Studies on the Active Constituents of Compound 48/80

George W. Read* and James F. Lenney

*Department of Pharmacology, University of Hawaii School of Medicine, Honolulu, Hawaii 96822.* *Received September 7, 1971*

Compound 48/80 was studied for its behavior during dialysis and gel filtration. The dialysis results indicate that there is a mixture of substances possessing histamine-liberating activity which has an average HCl salt molecular weight of 1300. The gel filtration data are in agreement with the dialysis results and further indicate that the active constituents range in free base molecular weight approximately from 700 to 1400. These molecular weights suggest that the degree of polymerization of the active constituents ranges from the tetramer to the octamer, with the average being the hexamer.

In 1949, Baltzly, *et al.*,[1] described a family of long-acting blood pressure depressing substances obtained by reacting equimolar concentrations of formaldehyde and *p*-methoxy-*N*-methylphenethylamine. They proposed that the products have the structure shown in Figure 1. By countercurrent distribution they separated them into fractions which appeared to contain the dimer, trimer, tetramer, and higher oligomers. They associated the hypotensive activity with the trimeric and tetrameric members of the family. They also reported that the *p*-methoxy-*N,N*-dimethylphenethylamine family was quite active.

In 1966, DeGraw, *et al.*,[2] confirmed the presence of the dimer in the reaction mixture by isolating it, converting it to the *N,N*-dimethyl derivative, and showing it to be identical with the *N,N*-dimethyl dimer synthesized by an alternate route. This compound had no depressor activity, in agree-

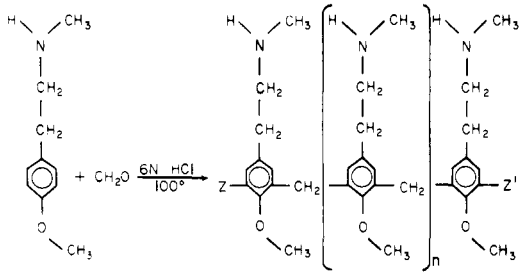

Figure 1. Reaction for the synthesis of compd 48/80 according to Baltzly, *et al.* Z and Z' may be H, $CH_2OH$, or $CH_2Cl$.

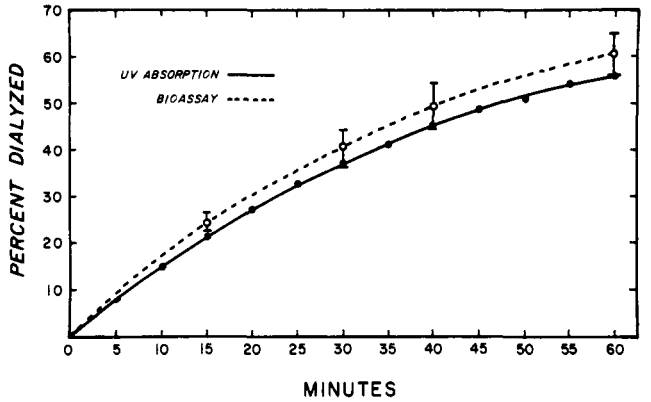


Figure 2. The rate of dialysis of compd 48/80 as detd by its hypotensive activity and absorption at 280 nm. The vertical lines represent ± the standard error at each point.

ment with the earlier finding. Similarly, DeGraw, *et al.*,[3] synthesized the *N,N*-dimethyl trimer and found it to be inactive, in contrast to the findings of Baltzly, *et al.* They concluded that the active constituents of the family must be higher molecular weight oligomers.

Since its discovery, a precipitate from this reaction mixture known as compound 48/80, has been shown to be a histamine liberator[4] and is extensively used in the study of this and related phenomena because of its high potency in most systems. We have studied the characteristics of this mixture during dialysis and gel filtration in an attempt to determine the molecular weights of the active constituents.

Figure 2 shows the rate of dialysis of compound 48/80 when followed by its hypotensive activity and by ultraviolet absorption. The half-time to dialyze the depressor activity was about 41 min, slightly less than the half-time for the uv-absorbing material. When this half-time for dialysis is compared to the half-times of substances of known mol wt (Figure 3), it can be seen that 41 min is equivalent to a mol wt of approximately 1300. This mol wt for the HCl salt would correspond to the hexamer.

When compound 48/80 was dialyzed for three 1-hr periods against a separate volume of $H_2O$ for each period, the activity that dialyzed each hour (relative to the activity present at the beginning of that hour) lessened with each succeeding dialysis (Table I). This indicates that the active material is not homogeneous, but instead is a population of substances of different molecular size. (As the population of active substances loses its smaller components, it takes longer for the remaining larger constituents to dialyze.)

Figure 4 shows the resolution of compound 48/80 achieved by passage through a Sephadex G-25 column. The void volume for this column was 91 ml; therefore some oligomers were probably large enough to be excluded from the bed matrix. In the same figure the hypotensive activity of each of the fractions is also plotted. It is apparent that the active constituents are of intermediate molecular size, while the larger constituents (smaller elution volumes) and smaller constituents (larger elution volumes) are inactive.

The fractions with elution volumes of 205, 194, 182, 168, and 153 ml were further purified by a second passage through

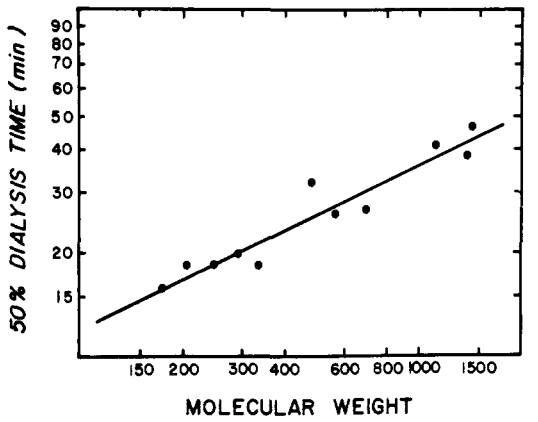


Figure 3. Calibration curve for detg mol wt by half-time to dialyze. From left to right the points represent the HCl salts of tyramine, norepinephrine, methoxamine, diphenhydramine, thiamine, quinacrine, emetine, curare, angiotensin, polymyxin, and bacitracin.

**Table I.** Escape of Hypotensive Activity during Successive Dialyses of Compound 48/80

| Sample | Amount in bag at start of period,[a] mg | Amount in sample,[b] mg | Per cent escaped[c] |
|---|---|---|---|
| Diffusate after first hr | 180.0 | 116.7 (112.5 ± 8) (120.9 ± 6) | 65 |
| Diffusate after second hr | 63.3 | 34.8 (32.8 ± 2.5) (36.8 ± 3.4) | 55 |
| Diffusate after third hr | 28.5 | 13.3 (12.5 ± 0.5) (14.1 ± 0.7) | 47 |
| Bag contents after third hr | | 18.7 (18.0 ± 1.7) (19.5 ± 1.9) | |
| | | 183.5 | |

[a] The amount of compd 48/80 present at the beginning of the second and third dialysis periods was calcd by subtracting from 180 the number of mg of hypotensive activity that escaped in the first and second periods. [b] The data in parentheses are the averages of the individual experiments and their standard errors. [c] The "per cent escaped" represents the quantity of compd 48/80 that dialyzed each period relative to the amount present at the beginning of that period.

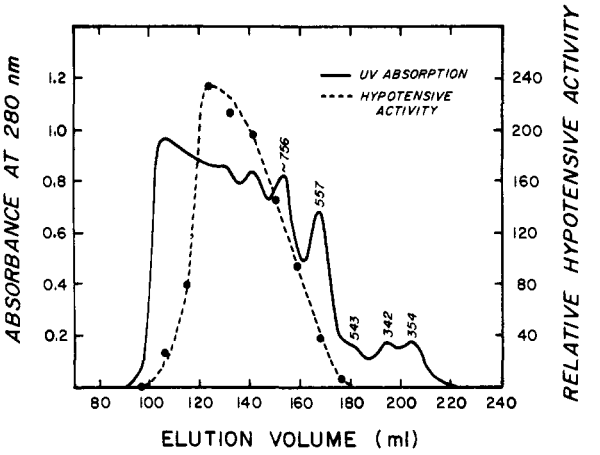


Figure 4. Elution pattern of uv-absorbing material and hypotensive activity obtained when 10 mg of compd 48/80 were chromatographed on a column of Sephadex G-25.

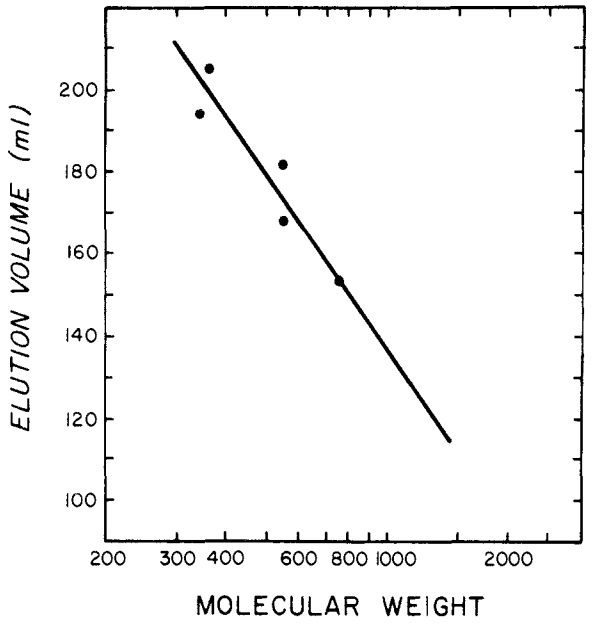


Figure 5. Relationship between mol wts of the free bases and elution volumes of the last 5 components of compd 48/80 to emerge from a Sephadex G-25 column.

Sephadex. When these purified fractions were analyzed in the mass spectrometer, free base mol wts of 354, 342, 543, 557, and 756, respectively, were obtained (the last value is uncertain because it is near the upper limit of the capability of the instrument). These mol wts are shown above their respective peaks in Figure 4. The mass spectra indicated that there was little or no cross-contamination between these 5 fractions.

When elution volumes were plotted against these mol wts, Figure 5 was obtained. Applying this calibration curve to Figure 4, it can be seen that the free base mol wts of the active constituents range approximately from 700 to 1400 with an average of about 1200. These free base mol wts would be appropriate for the tetramer through the octamer, with the average again being the hexamer. The G-25 column was also calibrated using peptides and other compounds unrelated to compound 48/80. Use of this calibration curve produced appreciably higher values for the mol wts of the active fractions. In any case, the dialysis and gel filtration data indicate that the most potent constituents are considerably larger than the trimer and tetramer postulated by Baltzly, *et al.*

When a different lot of compound 48/80 was chromatographed on the same Sephadex G-25 column, a similar elution pattern was obtained. However, the relative heights of the various peaks differed considerably from the uv-absorption curve shown in Figure 4. In addition, there was a difference in potency between the 2 lots.

Paper chromatograms of 100 $\mu$g of compound 48/80 usually showed 7 or 8 distinct spots, with minor components of intermediate mobility producing a streaking effect. No unreacted *p*-methoxy-*N*-methylphenethylamine was detected. The last 5 fractions emerging from Sephadex G-25, after repurification on Sephadex, were also analyzed by paper chromatography. The fractions emerging at elution volumes of 205 and 181 ml showed 1 major spot and 1 trace contaminant. The other 3 fractions showed 2 major components plus 1 or more minor components. It is possible that the additional components are homologs of the main component.

In some of the published studies employing compound 48/80, concentrations have been expressed in terms of molarity. It is not clear what assumptions were made to convert the concentrations from mass units to moles, but there are at least two possible sources of error. First, an appreciable amount of the product is inactive, and second, the average mol wt of the active portion is considerably higher than that of the trimer or tetramer.

## Experimental Section

**Dialysis.** A modification of the method of Craig, *et al.*,[5] was used. A length of dialysis tubing (27/100, Union Carbide, 22 mm in diameter) was pulled over a glass tube (12 mm o.d. by 85 mm long), and rubber rings were inserted at the ends so as to leave a uniform space between the glass tube and the dialysis membrane. An aqueous soln (9 ml) contg 1 mg of compound 48/80/ml was then injected through one of the rubber rings into the concentric space; the excess dialysis tubing at each end was then tucked inside the glass tube and held in place with snugly fitting corks. This dialysis assembly was then rotated in 900 ml of magnetically stirred distd $H_2O$ for periods of 15, 30, 40, and 60 min (2 separate runs for each time period) and the diffusates were then assayed. Compound 48/80 and the HCl salts of compds of known mol wt (tyramine, norepinephrine, methoxamine, diphenhydramine, thiamine, quinacrine, emetine, curare, angiotensin, polymyxin, and bacitracin) were also dialyzed in the same manner, their rates of dialysis being followed spectrophotometrically. In another series of experiments (performed twice), 9 ml of an aqueous soln contg 20 mg of compound 48/80/ml was dialyzed for 3 hr against a fresh vol (900 mg) of dist $H_2O$ each hour.

**Gel Filtration.** A Sephadex G-25 (Medium) column 111 × 1.5 cm was employed. A soln of 0.03 *N* AcOH adjusted to pH 3.0 with HCl was passed through the column at a flow rate of 12 ml/hr. (A low pH was used to suppress the ionization of the carboxyl groups of the Sephadex.) Samples (10 mg) were added in a vol of 1 ml of the eluant. The effluent was continuously monitored at 280 nm, and 9-ml fractions were collected.

**Mass Spectrometry and Paper Chromatography.** The last 5 fractions obtained from the gel filtration sepns were further purified by a second passage through the Sephadex G-25 column (a Sephadex G-15 column was used for the last 2 peaks). Only the central portion of each peak was used in the rerun on Sephadex and for the final lyophylization. A portion of each purified sample was analyzed in a Hitachi-Perkin Elmer RMU6E mass spectrometer. Another portion was chromatogd overnight by descending flow on Whatman No. 1 paper using *n*-BuOH–AcOH–$H_2O$ (12:3:5) as the solvent system. The dried chromatograms were sprayed with freshly prepared 0.2% ninhydrin in acetone.

**Bioassay.** Wister-derived (MW-3) male rats (300–600 g) were anesthetized with pentobarbital sodium (50 mg/kg, Abbott) and monitored on a Grass Model 7 polygraph. Arterial pressure was measured with a liquid-pressure transducer connected by a cannula to the right femoral artery. Heparin sodium (10 mg/kg, Nutritional Biochemicals) and the fractions to be assayed were given through a cannula inserted into the right femoral vein.

To determine the activities of the various fractions, a dose–response curve for compound 48/80 was prepared. Mean blood pressures of 40 rats were noted just before the administration of a dose, and again 5 min later. The depressor responses of the various fractions were measured in the same way, and their activities, derived from the dose–response curve, were expressed as equivalent micrograms of 48/80. For each bioassay of an active fraction, no fewer than 2 (usually 3–5) animals were used.

**Acknowledgment.** Two samples of compound 48/80 were obtained from Burroughs Wellcome & Co., Inc., through the generosity of Dr. Richard Baltzly. An authentic sample of *p*-methoxy-*N*-methylphenethylamine was obtained from Dr. Edgar F. Kiefer. We also wish to acknowledge the suggestions and criticisms of Dr. Edgar F. Kiefer, the mass spectrometry analyses and interpretations performed by Sister Mary Roger Brennen, and the technical assistance of Mrs. Charlotte S. Oda. This work was supported by grants from the University of Hawaii Research Council and the National Institutes of Health (GM-15198).

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# The Synthesis of 5-Hydroxymethyl-, 5-Acetoxymethyl-, and 5-Methylmercapto-7,12-dimethylbenz[*a*]anthracenes, and of 5,7,12-Trimethylbenz[*a*]anthracene†


Melvin S. Newman* and Robert F. Cunico

*Evans Chemistry Laboratory of Ohio State University, Columbus, Ohio 43210.*

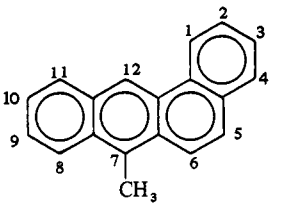
*Received September 11, 1971*


The evidence for assuming that the 5 position of 7-methylbenz[*a*]anthracene and 7,12-dimethylbenz[*a*]-anthracene is important in the metabolism by which these hydrocarbons induce the formation of cancer is presented. The syntheses of 5-hydroxymethyl-7,12-dimethylbenz[*a*]anthracene (**5**), 5-acetoxymethyl-7,12-dimethylbenz[*a*]anthracene (**6**), 5,7,12-trimethylbenz[*a*]anthracene (**7**), and 5-methylmercapto-7,12-dimethylbenz[*a*]anthracene (**8**) are described. Since none of these compounds is carcinogenic, they probably do not enter into the carcinogenic metabolic pathway by which 7,12-dimethylbenz[*a*]anthracene produces cancer.

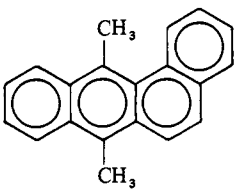

Some years ago a cooperative program designed to find out more about the carcinogenic activity of 7-methylbenz[*a*]anthracene (**1**) was started by our group at Ohio State University and Drs. J. A. and E. C. Miller at the University of Wisconsin. 7-Methylbenz[*a*]anthracene (**1**) was selected because, in the laboratories of several investigators, each of whom had studied all of the monomethyl derivatives of benz[*a*]anthracene, **1** proved to be by far the most active carcinogen.[1-4] The hypothesis was proposed that **1** was the most active of all of the monomethylbenz[*a*]anthracenes because of the presence of a 7-Me group, the position in the parent aromatic hydrocarbon most easily attacked by chemical reagents.[5] It was reasoned that when a benz[*a*]anthracene is unsubstituted at the 7 position, the host is able to deactivate the compound by means of reactions which take place at that position. However, when the 7 position is blocked by Me, the compound cannot thus be deactivated. Hence, more compound may be available for reaction at another position in such a way that cancer results.‡

As one approach to find out at which position(s) metabolism leading to cancer occurred, we undertook to synthesize the 11 monofluoro-7-methylbenz[*a*]anthracenes. We reasoned that F at a position not involved in carcinogenic metabolism might not greatly affect the carcinogenic activity of **1**. However, if F were placed at such a critical position, then activity would be greatly diminished.[6,7] Furthermore, since 7,12-dimethylbenz[*a*]anthracene (**2**) is a more active carcinogen than **1**, the synthesis of monofluoro derivatives of **2** was also deemed desirable.

The explanation as to why **2** is more active than **1** is difficult to give solely in terms of our original hypothesis. Since 12-methylbenz[*a*]anthracene is carcinogenic without having



**1**



**2**

a blocking group at 7 (as are 6-methyl- and 8-methylbenz[*a*]anthracenes) it may be argued that since the 12-Me group is situated at one of the meso positions of the anthracene portion of benz[*a*]anthracene somehow it helps to block the detoxifying metabolism and hence allow the compound to be present long enough for the carcinogenic process to occur. To the extent that 12-methylbenz[*a*]anthracene is carcinogenic whereas the parent hydrocarbon is not, the greater activity of **2** than **1** is explained. A further explanation for the greater activity of **2** as compared to **1** may have a steric origin. The presence of a Me group at 12 causes the molecule **2** to be more sterically crowded than **1**. Since this is undoubtedly a significant factor§ **2** is probably more reactive toward both chemical and biological reagents than **1** and the cancer-initiating reaction may be favored. This steric factor may be used to account for the carcinogenic activity of 12-methylbenz[*a*]anthracene by assuming that its cancer-producing metabolism is favored relative to the deactivation metabolism.

We have prepared all of the monofluoro-7-methylbenz[*a*]-anthracenes[9-14,#] except the 11- and 12-F compounds**,†† and submitted samples for testing.‡‡ Of these, only 5-

†This work was supported by Grant CA-07394 of the National Institutes of Health.

‡Of the other monomethyl derivatives, only the 6, 8, and 12 isomers have appreciable activity. The activity of the 6- and 8-Me isomers may be explained on the basis of our hypothesis because Me groups in the peri positions adjacent to 7 would be expected to have a steric effect (see text) which would delay metabolic deactivation at the 7 positions. The activity of the 12-Me isomer is harder to explain. Probably the main reason is related to a different kind of steric effect: an effect on the whole molecule which has a much higher ground state energy due to the molecular overcrowding caused by the 12-Me group.

§See Frisch, *et al.*,[8] for a discussion of the principle involved.

#4-F-7-methyl,[9] 9-F and 10-F-7-methyl,[10] 3-F- and 6-F-7-methyl,[11] 2-F-7-methyl,[12] and 8-F-7-methyl.[14]

**The synthesis of 12-fluoro-7-methylbenz[*a*]anthracene has been reported by Blum, *et al.*,[15] but to our knowledge no biological testing has been divulged. Dr. E. D. Bergmann (private communication) stated that he was to prepare 11-fluoro-7-methylbenz[*a*]anthracene.

††Dr. James A. Miller has informed me that he is preparing a paper which will summarize the carcinogenic activity of a large number of halogenated (mainly fluorinated) benz[*a*]anthracenes.

‡‡All comments concerning carcinogenic activity (or lack of it) obviously pertain only to cancer induced by the techniques used by the Millers, and where pertinent, C. Huggins and coworkers.