Subsequently, the HPLC fractionation was repeated under the same conditions but with the collection of smaller fractions which were tested for mutagenicity. The results for 4a (Fig. 1) show two peaks with mutagenic activity. These peaks were later shown to coincide with samples of 1,6- and 1,8-dinitropyrene (*6*). The mutagenicity of these compounds is extremely high (*7*), thus explaining the lack of detectable response in the mass spectrometer analysis.

It has been established that several photocopying processes are associated with mutagenic compounds that can be detected with the *Salmonella* assay. Evidence by Rosenkranz *et al.* (*3*) indicates that dinitropyrenes (present as impurities in the carbon black, the toner colorant) are the principal mutagens in selected toners of manufacturer 1. They found that through modification of the carbon black manufacturing process, it is possible to reduce substantially the nitropyrene content of the carbon black and thereby correct the problem.

Our results indicate that the mutagenic compounds found in the aromatic fraction of the extracts are the same for copies from manufacturers 1, 2, and 4. Manufacturers 3 and 4 have, in addition, mutagenic compounds present in their toners and copies that are different from nitropyrenes. We suggest that these compounds may be related to nitrogen-containing aromatic dyes, some of which have been shown to be mutagenic in the absence of metabolic activation (*8*).

The potential health hazards of mutagenic components in copies and toners are difficult to evaluate (*9*). It is not known whether the different compounds can volatilize in the copying process or from copies or can be transferred to the skin from the copies when they are handled. Neither is it known what will happen to the mutagens when copies are recycled in paper manufacturing or treated as waste material and possibly incinerated.

Nitroarenes, similar to or the same as those responsible for the mutagenic activity in some of the copies and toners, may also be present as common air pollutants. Their presence may explain why a substantial part of the mutagenic activity of combustion emissions and airborne particulate matter detected by the *Salmonella* assay does not require rat liver activation (*2, 10*). Precursors to these compounds, polycyclic aromatic hydrocarbons and nitrogen oxides, are both present in combustion emissions and polluted air, and they have been shown to react to nitroarenes (*11*).

Note added in proof: After the submission of this report, we verified that copies made from the modified carbon black toners of manufacturer 1 (*3*) did not yield mutagenic extracts.

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References and Notes

1. B. N. Ames, J. McCann, E. Yamasaki, *Mutat. Res.* **31**, 347 (1975).
2. G. Löfroth, in *International Symposium on Health Effects of Diesel Engine Emissions* (Environmental Protection Agency, Washington, D.C., in press).
3. H. S. Rosenkranz, E. McCoy, D. R. Sanders, M. Butler, D. K. Kiriazides, R. Mermelstein, *Science* **209**, 1039 (1980).
4. E. L. Wynder and D. Hoffman, *J. Air Pollut. Control Assoc.* **15**, 155 (1965).
5. The combined gas chromatography-mass spectrometry and the mass spectrometry analyses were performed by B. Jansson at the Special Analytical Laboratory of the Swedish Environment Protection Board on a Hewlett-Packard 5930A-5933A system.
6. A series of nitropyrene samples were provided by the Xerox Corporation.
7. The mutagenic response in TA98 was 300, 450, and 750 revertants per nanogram for 1,3-, 1,6-, and 1,8-dinitropyrene, respectively.
8. B. N. Ames, H. O. Kammen, E. Yamasaki, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2423 (1975).
9. All manufacturers mentioned in Table 1 were informed about the presence of mutagenic components in copies and toners during the first half of 1979.
10. M. Møller and I. Alfheim, *Atm. Environ.* **14**, 83 (1980); G. Löfroth, *Chemosphere* **7**, 791 (1978).
11. J. N. Pitts Jr., *et al., Science* **202**, 515 (1978).

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Nitropyrenes: Isolation, Identification, and Reduction of Mutagenic Impurities in Carbon Black and Toners

Abstract. *Extracts of selected xerographic toners and copies were found to be mutagenic in the* Salmonella *assay. The activity was independent of the xerographic hardware and process and was traced to nitropyrenes present as impurities in the carbon black, the toner colorant. Manufacturing process changes resulted in a substantial reduction of the nitropyrene content of the carbon black and thus in the mutagenicity of the corresponding toners. Nitropyrenes are potent frameshift mutagens, and possible mechanisms for their biological action are discussed.*

During an investigation of the various sources of mutagenicity (*1*) in the Stockholm environment, Löfroth *et al.* (*2*) detected genetic activity in the acetone and dimethyl sulfoxide (DMSO) extracts of selected xerographic copies and toners. The mutagenic activity calculated from the extracts of types B and H toners (*3*) and the copies made (*4*) with these toners ranged from 1.2×10^5 to 12×10^5 revertants per gram of toner in *Salmonella* tester strain TA98 without microsomal activation (*5*). We initiated a major collaborative effort to detect the source of the mutagenic signal, to identify the chemical (or chemicals) responsible for it, and to reduce it.

We confirmed Löfroth's observations (*2*) on toner B and copies made using toner B, but the use of the same procedure on toner H did not result in a reproducible demonstration of mutagenicity (*3*). When we used a modified procedure (*6*), linear dose responses were generally observed. The activity was greatly decreased or even eliminated when microsomal preparations were included in the assay (*1*). The highest responses were observed with *Salmonella* tester strains TA98 and TA1537, lower values with TA1538, only occasional responses with TA100, and consistently no activity with strain TA1535.

In tracing the source of the activity, it became essential to determine whether the xerographic equipment, the process, or both influenced the mutagenic response. Extracts of copies produced on the Xerox 3100 machine showed a five- to sixfold increase in mutagenicity, while equivalent extracts derived from image-free paper that had passed through the copying process had values comparable to the spontaneous frequency (Table 1, experiments 1 and 2). Use of the same toner formulation in a Xerox 660 copier (Table 1, experiment 3) and a Xerox 2300 machine (Table 1, experiment 4) with alternative development and fusing subsystems, respectively, produced copy extracts that showed similarly elevated mutagenic activity. In contrast, no activity was detected in the extracts of copies made with the Xerox 9200 toner formulation in the same 2300 machine (Table 1, experiments 5 and 6). Thus, from two separate sets of experiments—(i) the same toner formulation used with different hardware and (ii) a different toner formulation used with the same hardware—the conclusion is reached that only differences in toner formulation influence the mutagenic response.

Confirmation of these findings was obtained by formulating a series of toners composed of four different polymers and

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Table 1. Results of *Salmonella* assay without activation of copy extracts generated under various conditions. Three copies (21.6 by 27.9 cm) with line print were cut into strips and placed in a flask with 20 ml of acetone. The paper absorbed approximately 10 to 15 ml of the solvent. The mixture was shaken and portions of the solution were used in the assay. The values shown represent 75-μl amounts of the above solution. The standard (*1*) *Salmonella* plate incorporation method was used.

Experiment	Toner	Hardware	Condition	*Salmonella* strain (revertants per plate)				
				TA1535	TA100	TA1537	TA1538	TA98
			Baseline					
1	3100	3100	Normal	27	133	42	58	146
2	3100	3100	Blank paper	19	112	29	7	38
			Same toner, different hardware					
3	3100	660	Normal	9	158	66	57	113
4	3100	2300	Normal	17	160	59	62	188
			Different toner, same hardware					
5	9200	2300	Unfused	14	101		13	27
6	9200	2300	Normal, fused	21	107		17	21
			*Blank (acetone)**	21 ± 5.5	116 ± 22	14 ± 5	11 ± 3.3	35 ± 4

*Mean ± standard deviation.

two different carbon blacks at the typical 10 percent loading and evaluating the corresponding DMSO slurries (*6*) for mutagenicity in the *Salmonella* assay. The results (Table 2) show that all four toners made from carbon black A yielded nonmutagenic slurries, while those containing carbon black B were mutagenic (*7*). The magnitude of the mutagenic signal was influenced by the polymer used in the toner; higher values were obtained in the presence of the vinyl polymers than with polyester. Although DMSO slurries of toners containing carbon black B were mutagenic under our experimental conditions, similar slurries of each raw material, that is, carbon black, polymer, and other individual ingredients, were not. This apparent paradox is due to competitive adsorption. Prolonged Soxhlet extraction of the carbon black with solvents such as toluene, however, produced solutions that exhibited mutagenicity. To exclude involvement of the toner fabrication process, we gently mixed (*8*) finely ground polymer and carbon black of the above formulations and assayed the resultant samples. In each instance, the dry blend containing carbon black B yielded mutagenic activity, although of slightly lower magnitude than the corresponding toner, while the blends containing carbon black A were genetically inert. These results demonstrated that the mutagenic activity was associated with the toners containing carbon black B.

Examination of the toluene extracts (*9*) of these two blacks proved informative in that the mutagenic activity from carbon black B was much higher than that of carbon black A (Table 3). The consistently high responses in tester strains TA98, TA1538, and TA1537 are indicative of frameshift mutations (*1*). The lack of stimulation and actual decrease in mutagenic activity on incubation in the presence of microsomal preparations (*10*) suggest the presence of direct-acting species not requiring enzymatic activation to express their mutagenicity. Unsubstituted polycyclic aromatic hydrocarbons (PAH) generally require metabolic activation to exert their mutagenicity in bacterial systems (*1*). Indeed, high-performance liquid chromatographic (HPLC) analysis, while indicating the presence of more than 50 compounds, showed very low concentrations of PAH. Benzo[*a*]pyrene, typically present at about 1 part per million (ppm) in ordinary carbon black, was found to be only 0.001 ppm. The concentration of pyrene, usually a major component of such extracts, was 0.06 ppm.

Table 2. *Salmonella* assay results (tester strain TA98 without activation) of toner slurries. A slurry of the toner in DMSO (5 mg/ml) was agitated at 30-minute intervals for 5 hours. The values shown represent a 100-μl portion of the toner slurries. Toners were fabricated from 90 percent polymer and 10 percent carbon black. Polymer 1 is a propoxylated bisphenol-A-fumarate, molecular weight, ~ 7000; polymer 2 consists of a ratio of styrene to 2-butylmethacrylate of 80 to 20 (percent by weight), molecular weight, ~ 28,000, plus 20 percent pentaerythritol tetrabenzoate (plasticizer); polymer 3 consists of a ratio of styrene to 1-butylmethacrylate of 58 to 42, molecular weight, ~ 70,000; polymer 4 consists of a ratio of styrene to 1-butylmethacrylate of 65 to 35, molecular weight, ~ 70,000, plus 10 percent polyvinylbutyral.

Toner	Revertants from slurries containing carbon black types (No. per plate)		
	A	B	B*
Blank/DMSO	15	15	12
Polymer 1	12	38	8
Polymer 2	10	108	11
Polymer 3	12	75	8
Polymer 4	11	65	

By combining HPLC fractionation with the microbial assay (*11*) we were able to focus quickly on the most active fractions. The toluene extract from carbon black B was divided by reverse-phase HPLC into three fractions, a PAH fraction and two polar fractions (*12*). The middle fraction contained about half the total weight yet nearly all of the mutagenic activity. Further separation resulted in a fraction that accounted for less than 3 percent of the weight of the total extract yet contained more than 80 percent of the total mutagenic activity. Its mass spectrogram (*13*) indicated a molecular ion of *m/z* (mass to charge) equal to 292, major fragment of mass 200, and loss of two successive 30-mass units. This evidence is consistent with the dinitro derivatives of either pyrene or fluoranthene. Since manufacture of carbon black B involves a nitration-oxidation step, appearance of nitrated PAH and disappearance of PAH appear to be plausible.

Next, samples of pyrene and fluoranthene were each nitrated and the reaction products chromatographed (*14*). The chromatograms of the nitrofluoranthenes (Fig. 1A), extract from carbon black B (Fig. 1B), nitropyrenes (Fig. 1D), and the most active subfraction (Fig. 1E) were compared. There is good agreement between peaks of the extract from carbon black B and the nitropyrenes (Fig. 1, B and D). Further, the two peaks of the most active subfraction (Fig. 1E) are common to both the extract from carbon black B and the nitropyrene chromatograms. In contrast, the pattern of peaks in nitrofluoranthenes (Fig. 1A) is quite different from that of the extract of carbon black B.

The identity of retention times in the HPLC chromatograms is evidence that nitropyrenes are present in carbon black B extract. To prove that they are responsible for the observed mutagenicity, we conducted two experiments. In one, the constituents of each of the two most potent peaks (Fig. 1E) were isolated from the carbon black B extract and their mutagenicities compared at the same concentrations to those of the corresponding peaks in nitropyrene (Fig. 1D, peaks 3 and 4). In the second experi-

ment, the mutagenicity of the entire nitropyrene region of the extract (Fig. 1B) was compared in a similar manner with that of a synthetic mixture made from the individual nitropyrenes. The coincidence in mutagenic activities found in the paired preparations in three strains is compelling evidence in support of the conclusion that nitrated pyrenes are responsible for the major portion of the mutagenicity of carbon black B extracts.

In order to permit unambiguous identification and structural assignment and to provide larger quantities of samples for biochemical investigation, the various nitro derivatives of pyrene attainable through direct nitration were synthesized (*15*) by modifications of the procedure of Vollman (*16*). Separation of the crude dinitropyrene reaction mixture proved to be difficult and was finally achieved through a combination of fractional crystallization and preparative HPLC to yield three dinitro isomers, including the hitherto unreported 1,3-dinitropyrene. Structural assignments (*13*) were made by means of ^{13}C nuclear magnetic resonance (NMR), and the 1,3-, 1,6-, and 1,8-dinitropyrenes were found to correspond to peaks 2, 3, and 4 in the nitropyrene chromatogram (Fig. 1D). The 1,3,6-trinitro- and 1,3,6,8-tetranitropyrenes, prepared from the dinitropyrene mixture and pyrene, respectively, correspond to peaks 5 and 6 in Fig. 1D.

Although nitro-containing chemicals, especially nitroheterocyclics, are widely used in human and animal medicine and despite the fact that nitroaromatics are presumably present in the environment as the result of industrial and automobile combustion (*17*), little is known of the structural requirements for the mutagenicity of these chemicals, especially the nitroaromatics. While nitroheterocyclics are mainly base-substitution mutagens (*18*), nitroaromatic chemicals, depending upon their molecular size, can induce either base-substitution (for example, 2-nitronaphthalene) (*19*) or frameshift mutations (for example, 2-nitrofluorene, 6-nitrobenzo[*a*]pyrene) (*19–21*). It has been assumed that the biologic activity of these chemicals is derived from their enzymic reduction to the corresponding hydroxylamino compounds which then react covalently with the base moieties of the DNA. Indeed, the mutagenic activity of such chemicals is greatly reduced when *Salmonella* tester strains deficient in nitroreductase are used (*19, 22*). It would seem that the frameshift mutagenic activity (that is, the predilection for strains TA98 and TA1538, but not TA1537) is due to a base-displacement reaction (*23*). This reaction results from the covalent attachment of the active intermediate to one of the heterocyclic bases of the DNA rather than an intercalation of the chemical between DNA base pairs since classically "pure" DNA intercalators (for example, proflavin, 9-aminoacridine, quinacrine) induce mutations in strain TA1537 only (*21, 24*).

Table 3. *Salmonella* assay results (tester strain TA98 without activation) of carbon black extracts. A 10-g quantity of a carbon black (*7*) was extracted with toluene for 48 hours. The extract was concentrated at low temperature and the solvent exchanged with DMSO (final volume, 1.0 ml). The values are expressed in micrograms of carbon black extracted. Subscripts refer to different batches of the type of carbon black.

Concentration (μg per plate)	Revertants in extracts of carbon black types (number per plate)					
	A_1	A_2	B_1	B_2	B^*_1	B^*_2
0	17	31	17	7	7	7
10.0	27		215	127	8	7
100.0	29	28	1,845	1,263	10	13
1,000.0	108	55	715	1,407	30	223
10,000.0	212	187			284	781

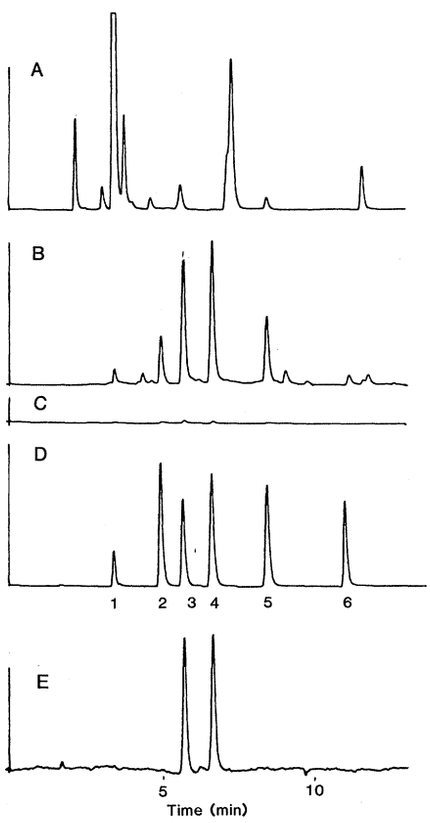

Fig. 1. HPLC chromatograms. (A) Fluoranthene nitration products. (B) Carbon black B, extract from 6 mg. (C) Carbon black B*, extract from 6 mg. (D) Pyrene nitration products. Peaks were identified as (1) 1-nitropyrene, (2) 1,3-dinitropyrene, (3) 1,6-dinitropyrene, (4) 1,8-dinitropyrene, (5) 1,3,6-trinitropyrene, and (6) 1,3,6,8-tetranitropyrene. (E) Carbon black B most active fraction. Data obtained on a Zorbax CN (35°C; 2 ml/min, 5 to 60 percent *i*-propanol-hexane; 400-nm ultraviolet detector).

The mutagenic activity of the nitropyrenes described is substantially higher than that of related nitro compounds and indeed is among the highest encountered. Up to five times higher mutagenic activities than listed in Table 4 are obtained in strains TA98 and TA98NR when resting bacteria are added to the plates. Presumably this reflects the metabolic state of these cells. The activity of 1×10^3 revertants per nanomole in strain TA98 obtained with 1-nitropyrene is 5×10^3 times higher than the results for 2-nitronaphthalene (0.2 revertants per nanomole, TA98 − S9) (*21*). The calculated activities for the three dinitropyrene isomers are even higher than that of 1-nitropyrene, ranging from 2.8×10^4 to 7.3×10^4 revertants per nanomole or 1.4×10^5 to 3.7×10^5 higher than for 2-nitronaphthalene. The mutagenic activity of 7.3×10^4 revertants per nanomole calculated for 1,8-dinitropyrene in strain TA98 is to be compared to 2.76×10^3 revertants per nanomole (TA98 + S9) for 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (*25*), heretofore considered the most mutagenic substance, and 376 revertants per nanomole for the related benzo[*a*]pyrene 7*b*,8*a*-diol-9*a*,10*a* epoxide (TA98 − S9) (*26*). The specific activity of the 1,3,6-trinitropyrene is in the same range as the 1,3-dinitro-isomer, while the tetranitro compound shows significantly lower activity, 7.7×10^3 revertants per nanomole. A similar pattern was observed in the nitrofluorenone series, where the addition of up to three nitro groups increased successively the mutagenic activity while the addition of the fourth nitro group resulted in a decreased signal (*21*). It is important to emphasize that the above comparisons reflect the mutagenicity in *Salmonella* of the pure nitropyrenes, which were present as impurities in the affected toners at a level of less than 10 ppm. Moreover, except for the

Table 4. Mutagenicity of various nitropyrenes for *Salmonella*. The standard (*1*) *Salmonella* plate incorporation method, except for the use of exponentially growing cultures, was used. The specific mutagenic activity listed has been calculated from the linear portion of the dose-response curves. The experimental values are omitted in the interest of space and will be published elsewhere.

Compound	Revertants for *Salmonella* strains (number per nanomole)					
	TA1535	TA100	TA1537	TA1538	TA98	TA98NR
1-Nitropyrene	0	225	500	260	1,050	950
1,3-Dinitropyrene	0	8,350	13,400	15,600	28,600	4,900
1,6-Dinitropyrene	0		33,300	12,100	36,350	37,850
1,8-Dinitropyrene	0		11,800	9,950	72,900	75,500
1,3,6-Trinitropyrene	0	5,750	20,100	15,650	31,400	28,330
1,3,6,8-Tetranitropyrene	0	750	3,300	1,350	7,700	5,200

1,3-dinitropyrene and 1,3,6,8-tetranitropyrene, the mutagenic activity was not significantly reduced when tested in the nitroreductase-deficient tester strain. This is in contrast to the behavior of other nitroaromatics (for example, the structurally related nitrofluorenones) which exhibit only modest mutagenic activities and which require a functional nitroreductase (*19, 21*).

These findings suggest that either the nitropyrenes are direct-acting mutagens or that they are "activated" by the bacteria by a mechanism not involving the classical nitroreductase. A direct action of these chemicals could result from intercalation between DNA base pairs which in turn could lead to frameshift mutations. However, as pointed out above, DNA intercalators are expected to induce mutations in strain TA1537 only. This was not observed with these chemicals. In addition, experiments with isolated DNA (calf thymus as well as covalently closed circular plasmid DNA) show no evidence for intercalation as determined by helix-to-coil thermal transition profiles (T_m), sucrose gradient centrifugation, and spectroscopy (*21*).

An alternative mechanism for a direct reaction with the cellular DNA involving covalent linkage is by analogy with the well-known diol epoxides (*27*). If 1,8-dinitropyrene is overlayed onto benzo[*a*]pyrene diol epoxide, it can be visualized that the nitrogen atom of the nitrogroup can play the role of the benzylic carbon atom. In either case, the resulting carbonium ion (or nitronium ion) is stabilized by resonance and an electron-rich oxygen atom. However, results with isolated DNA provide no evidence for a covalent reaction between the nitropyrenes and DNA as determined by T_m sucrose gradient centrifugation, and susceptibility to S_1 nuclease (*21*).

The above speculations lead to the suggestion that reduction of the nitro functions of nitropyrenes is required for mutagenic activity and that this is accomplished by a bacterial enzyme that is different from the nitroreductase that acts on nitroheterocyclics and some nitroaromatics. In support of this hypothesis, we have recently succeeded in isolating *Salmonella* tester strains that have retained their permeability to large molecules and are resistant to the mutagenic actions of dinitropyrenes and yet can be mutagenized by other nitro-containing chemicals (*21*). Experiments are currently in progress to characterize this possibly new enzymic activity and to determine whether it is unique to bacteria. In this connection it should be pointed out that the level of activity of the classical nitroreductase is much greater in bacteria than in mammalian cells, an explanation that has been given for the high mutagenicity of nitro-containing chemicals without correspondingly high carcinogenic activity (*1, 28*) or even a lack of carcinogenicity. It is also noteworthy that the mutagenic activity of the nitropyrenes is drastically reduced when they are tested in TA1978, a $UvrB^+$ (*29*) analog of strain TA1538 (*21*). It has been shown that while the Uvr (*29*) phenotype does not affect the expression of the frameshift activity of simple intercalators, it has a profound effect on the expression of reactive frameshift mutagens, that is, those forming covalent products with DNA (*30*). These findings lead to the conclusion that nitropyrenes are metabolized by the bacteria to intermediates that are linked covalently to DNA and that are susceptible to excision by uv-nuclease (*29*).

We have compiled the health records of Xerox employees (research and development, toner manufacturing, and machine service personnel) exposed to xerographic toners. Toners have been in production in quantity only since the late 1950's and the above cited carbon black B was first used in toner in 1967. Our analysis of these health records revealed no signs of health effects or clustering of any type (*31*). Xerox Corporation intends to continue medical surveillance of its employees. Mortality and morbidity studies of those who have worked with carbon black over several decades (*32*) have shown that there were no adverse health effects. The number of workers exposed to materials such as carbon black B is too small for a separate epidemiological analysis.

When the nitropyrenes were identified as the major mutagenic impurities present in the carbon black B, a plan for their substantial reduction or elimination became feasible. Since carbon black production is a highly refined and controlled process, the conditions were adjusted to reduce the nitropyrenes extractable by toluene by a factor of 50 to 200, as demonstrated by HPLC analysis of an extract of the modified carbon black B* (Fig. 1C). At these lower levels of dinitropyrenes, the mutagenicity of the modified carbon black B* extract is in the same range as that of carbon black A (Table 3). Toners formulated from the modified carbon black B do not give rise to a mutagenic slurry (Table 2) (*6*). The modifications incorporated into current manufacturing methods of carbon black B for use in toner production resulted in a black that is essentially identical to the former production material except for substantially reduced levels of nitropyrenes. This modified carbon black B* is being used by the various Xerox toner manufacturing plants.

Nitroarenes like nitropyrenes may be ubiquitous in the environment. Pyrene is a common PAH and is formed in most incomplete combustion processes while nitrating agents emanating from natural and man-made sources are widespread. The facile reaction of PAH to form nitrated derivatives has been documented (*17, 33, 34*). Likely sources of nitroarenes in the environment may include incinerators, coal-fired power plants, polluted air, and exhausts from internal combustion engines.

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References and Notes

1. B. N. Ames, J. McCann, E. Yamasaki, *Mutat. Res.* **31**, 347 (1975); J. McCann, E. Choi, E. Yamasaki, B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 5135 (1975); J. McCann and B. N. Ames, *ibid.* **73**, 950 (1976); B. N. Ames, *Science* **204**, 587 (1979).
2. G. Löfroth, E. Hefner, I. Alfheim, M. Møller, *Science* **209**, 1037 (1980).
3. Type B toner is used in the Rank Xerox 3600 copier, and type H in Rank Xerox 3100 copier.
4. In a normally operating xerographic copier or duplicator, 25 mg of toner are present on a typical copy of a typed sheet 21.6 by 27.4 cm.
5. G. Löfroth, personal communication.
6. The addition of polymer to a solution of a mutagenic chemical (for example, nitropyrene) resulted in decreased revertant counts and a leveling of the activity versus mutagen concentration slope. This reduction in mutagenic activity appears to be influenced by polymer solubility. Whether the dissolved polymer reduces the effective concentration of the mutagenic chemical in solution or is encapsulating or hindering the diffusion of the chemical into the bacterial cells remains to be determined. The modified procedure adopted consisted of preparing a slurry of toner, 5 mg/ml in DMSO, and agitating it for 30 seconds at 30-minute intervals for 5 hours. In the *Salmonella* assay we used 50-, 100-, and 200-μl amounts of the slurry.
7. Carbon black A is a medium color furnace black manufactured by Columbian Chemicals Division of Cities Service Company and is marketed under the name of Raven 5750. Carbon black B is a pelletized long flow furnace black manufactured by the Special Blacks Division of Cabot Corporation and is marketed under the name of Black Pearls L.
8. Mixing conditions: twin-shell blender without intensifier agitated at room temperature for 30 minutes.
9. Extraction conditions: 48-hour Soxhlet extraction using all glass equipment, coarse porosity glass thimble (Ace Glass, Inc., No. 68B-04) and shielded from the light.
10. Microsomes were prepared from the livers of Aroclor 1254–induced rats (F344N, male Fischer, 25 mg of protein per milliliter).
11. E. V. Donahue, J. McCann, B. N. Ames, *Cancer Res.* **38**, 431 (1978).
12. Reverse-phase chromatography was chosen to minimize the possibility of irreversible adsorption on the column. The toluene in the extract solution was concentrated by vortex evaporation (50°C, 50 torr) and the liquid residue taken up in acetonitrile. This was eluted through a Waters C-18 SepPak with acetonitrile and the eluate was concentrated and chromatographed to furnish three fractions. The SepPak was backflushed with tetrahydrofuran. No mutagenicity was found in this solution so that in subsequent chromatography it was assumed that all mutagens were elutable with acetonitrile.
13. Mass spectra data were obtained on a Nuclide Corporation 12-90G instrument with a temperature-programmed solids inlet probe (source temperature 250°C, ionizing voltage 70 eV, ionizing current 100 μA, beam current 105 A/μg, and resolution 1:1000). The ^{13}C-NMR data were obtained at 20.1 MHz with complete proton broadband decoupling on a Bruker WP-80 pulsed Fourier-transform spectrometer (110°C with concentrations of approximately 1 percent by weight in 99.5 percent DMSO-d_6). The ^{1}H measurements were made at 250 MHz on a Bruker WM-250 instrument.
14. Nitric acid (2 ml, 70 percent) was added dropwise to about 10 mg of fluoranthene or pyrene in the absence of solvent. The reaction mixture was filtered using glass frit washed with water and cold methanol and dissolved in methylene chloride.
15. W. H. H. Gunther, S. Kaplan, J. Van-Dusen, F. Elder, D. Evans, R. Mermelstein, in preparation.
16. H. Vollman, H. Becker, M. Corell, H. Streeck, *Justus Liebigs Ann. Chem.* **531**, 1 (1937).
17. J. N. Pitts, Jr., *Philos. Trans. R. Soc. London Ser. A* **290**, 551 (1979).
18. Y. Tazima, T. Kada, A. Murakami, *Mutat. Res.* **32**, 55 (1975); C. Y. Wang, K. Muraoka, G. T. Bryan, *Cancer Res.* **35**, 3611 (1975); H. S. Rosenkranz, W. T. Speck, J. E. Stambaugh, Jr., *Mutat. Res.* **38**, 203 (1976).
19. H. S. Rosenkranz and L. A. Poirier, *J. Natl. Cancer Inst.* **62**, 873 (1979).
20. Y. Y. Wang, S. M. Rappaport, R. F. Sawyer, R. E. Talcott, E. T. Wei, *Cancer Lett.* **5**, 39 (1978).
21. H. S. Rosenkranz, R. Mermelstein, E. C. McCoy, unpublished results.
22. H. S. Rosenkranz and W. T. Speck, *Biochem. Biophys. Res. Commun.* **66**, 520 (1975); H. S. Rosenkranz, *Biochem. Pharmacol.* **26**, 896 (1977).
23. A. F. Levine, L. M. Fink, I. B. Weinstein, D. Grunberger, *Cancer Res.* **34**, 319 (1974).
24. J. McCann *et al.*, in (*1*); W. T. Speck and H. S. Rosenkranz, *Mutat. Res.* **77**, 37 (1980).
25. T. Sugimura and M. Nagao, *Crit. Rev. Toxicol.* (1979), p. 189.
26. T. Kuroki, in *Polycyclic Hydrocarbons and Cancer*, H. B. Gelboin and P. O. P. Ts'o, Eds. (Academic Press, New York, 1978), vol. 2, p. 123.
27. D. M. Jerina, H. Hagi, R. E. Lehr, D. R. Thakker, M. Schaefer-Ridder, J. M. Karle, W. Levin, A. W. Wood, R. L. Chang, A. H. Conney, in *ibid.*, vol. 1, p. 173.
28. J. McCann and B. N. Ames, in *Origins of Human Cancer*, H. H. Hiatt, J. D. Watson, J. A. Winsten, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1977), book C, p. 1431.
29. The usual tester strains (TA1535, TA1537, TA1538, TA100, and TA98) have the *UvrB* genotype which is expressed in an enzyme deficiency (uv-nuclease). This deficiency, the product of the *UvrB* gene, renders the bacteria sensitive to ultraviolet light and to some chemical mutagens that form adducts with DNA.
30. B. N. Ames, in *Mutagenic Effects of Environmental Contaminants*, H. E. Sutton and M. I. Harris, Eds (Academic Press, New York, 1972), p. 57; B. N. Ames, F. D. Lee, W. E. Durston, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 782 (1973).
31. C. DeMarco, unpublished data.
32. J. McD. Robertson and T. H. Ingalls, "A mortality study in United States carbon black workers 1935-1974," paper presented at the Akron Rubber Group Symposium, Akron, Ohio, 1979; W. A. Crosbie, R. A. F. Cox, J. V. LeBlanc, D. Cooper, *ibid.*
33. P. B. D. de la Mare and J. H. Ridd, in *Aromatic Substitution, Nitration and Halogenation* (Academic Press, New York, 1969), p. 187.
34. M. M. Hughes, D. F. S. Natusch, M. R. Schuve, D. R. Taylor, paper presented at the Fourth International Symposium on Polynuclear Aromatic Hydrocarbons, Columbus, Ohio, 1979.

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Cells Isolated from the Embryonic Neural Retina Differ in Behavior in vitro and Membrane Structure

Abstract. *Several subpopulations of cells were isolated from trypsin-dissociated embryonic (14 days) chick retinas. The cells of each subpopulation differed in associative behavior measured by cell aggregation and stationary culture assays and in glycoproteins that contain glucosamine. Freeze-fracture analysis showed that these populations also differed in intramembrane particle content.*

During embryogenesis, the neural retina develops from a simple epithelium into a multilayered structure in which each cell layer is discrete and restricted to specific functions. The formation of the layers could be considered a result of associative differences between the different cell types of the retina. Several studies have shown that soluble factors promote the tissue-specific adhesion of retina cells to each other (*1*). However, none of these materials discriminates between cells in the tissue. Rutishauser *et al.* (*2*) demonstrated the presence of a material on cells of retina and other neural tissues that appears to affect fasciculation of axonal outgrowths. Although this is clearly relevant to an aspect of retina organization, it is not clear if it is related to the formation of the cellular layers, which are detectable before the formation of the plexiform layers. In order to examine differences among the cells directly, we have been attempting to develop techniques to isolate specific cell types from the neural retina of the embryonic chick. Of the procedures we considered (*3*), we found that a buoyancy equilibrium method allowed us to isolate four cell populations that differ in associative behavior and membrane structure.

Embryonic (14 days) chick retinas, which contained all of the cell layers, were dissociated with 1 percent trypsin in calcium- and magnesium-free Tyrode's solution (*4*). The single cells were then resuspended in Eagles minimal essential medium containing 10 percent fetal calf serum (*5*). The suspension was divided among three solutions of Percoll (*6*) in medium and then adjusted to final densities of 1.019, 1.034, and 1.060 g/ml. These suspensions were incorporated into a step gradient with a top layer of medium and a cushion of 1.090 g/ml. The total volume of the gradient was 190 ml, and it contained up to 10^9 cells. The gradient was placed in a HS-4 rotor and centrifuged at 770*g* for 1 hour in a Sorvall RC-2B centrifuge equipped with a slow acceleration modification. Cells were collected from the interfaces, and the populations were labeled 1, 2, 3, and 4 in order of increasing buoyant density. The cells in each fraction were counted and classified by size. The four fractions contained 35 percent of the original cells; the rest were between the interfaces. Phase-contrast micrographs of the populations are given in Fig. 1, a to d, and the relative proportions of the cells are summarized in Table 1.

The cells in fraction 1 are large—78

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