rons so that satiety would not be inhibited and the animal would not feed.

On the other hand, NE infused into the lateral hypothalamic area attenuates eating in the deprived animal (*3, 9*), presumably by inhibiting neurons that could be responsible for activating feeding. Consequently, the presence of a nutrient in the duodenum should evoke the synaptic release of NE from noradrenergic neurons in the lateral hypothalamus and thus prevent feeding. This is what occurs. Thus, the anatomically distinct output of NE from the medial noradrenergic neurons that activate satiety as well as lateral hypothalamic neurons yields the same functional result.

The neuronal pathway that affects both sets of hypothalamic cells in harmony may be the afferent vagal pathway, which arises from the gastrointestinal tract and projects to the diencephalon (*24*). This hypothesis would account for the finding (*10*) that a lesion placed in this lateral hypothalamic area eliminates the satiating effect of an intraduodenal infusion of a nutrient. Finally, cholecystokinin, a peptide-containing gut hormone implicated in satiety also differentially affects the release of NE from sites in the rat's hypothalamus (*25*). This would suggest that the suppression of feeding by cholecystokinin may be due to the dual action of the peptide on noradrenergic neurons in both the satiety and feeding systems in the animal's hypothalamus.

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13. A 25-cm length of polyethylene-50 tubing was fitted at the duodenal end with heat flanged collars between which silk suture was passed and knotted to hold the catheter in place. A 1-cm^2 piece of Silastic sheeting was cemented to the catheter with epoxy (Devcon) at the level of the gastric incision. A midline incision was made through the abdominal musculature, the catheter was inserted through a 1-mm incision in the stomach wall, and then subsequently attached to the ventral wall of the stomach by two silk sutures placed around the ends of the collars. Externally, the Silastic sheet was attached to the surface of the stomach by purse-string suture. The polyethylene catheter was brought out of the peritoneal cavity and drawn subcutaneously to the caudal portion of the head, where it was attached by Cranioplastic cement to the skull. A 1-ml bolus of 0.9 percent saline was infused through the catheter every day to maintain its patency.
14. Standard procedures were used [R. D. Myers, in *Methods in Psychobiology*, R. D. Myers, Ed. (Academic Press, London, 1971), vol. 1, pp. 247–280] in which a 23-gauge needle tubing, cut to a length of 1.5 cm, was cemented in place.
15. The NE salt was dissolved in a concentration of 3.5 μg/μl in an artificial cerebrospinal fluid containing the chloride salts of Na^+, K^+, Ca^{2+} and Mg^{2+}. The microinjector needle was 27-gauge stainless steel tubing connected with polyethylene-20 tubing to a 50-μl syringe (Hamilton) mounted on an infusion pump (Harvard). The volume of 0.75 μl was delivered to each hypothalamic site over an interval of 35 seconds.
16. We did not test to see whether NE injected into these "negative" sites would inhibit feeding, because the integrity of its tissue for subsequent perfusion studies was essential. Such an experiment is equally important.
17. A reactive site was so categorized if the previously fed animal consumed 1.0 g or more of freshly ground Wayne Lab Blox within 15 minutes after the microinjection of the NE solution.
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Mutagenic Activity in Photocopies

Abstract. *Extracts from several different photocopies were mutagenic in the Ames* Salmonella *assay. The mutagenic behavior was similar for extracts from copies and corresponding toners indicating that toners are directly responsible for the mutagenicity. The mutagenicity is caused by at least two classes of compounds which may be present either alone or in combination in any toner.*

We report that copies produced and toners used in several photocopying machines contained compounds that are mutagenic in the Ames *Salmonella* assay (*1*).

Extracts of copies with print and extracts of toners used in the machines were found to be mutagenic in the *Salmonella* tester strain TA98 in the absence of rat liver metabolic activation (Table 1). The mutagenic response decreased by addition of S9 from rat liver, but the presence of NADP$^+$ (nicotinamide adenine dinucleotide phosphate) in the S9 mix was not essential for this decrease. Heat-inactivated S9 mix did not decrease the mutagenic response. The mutagenicity was also detectable with the tester strains TA1538 and TA1537. No mutagenicity could be detected with TA1535, whereas a weak response was seen with TA100. Extracts of the plain photocopy papers did not show any detectable mutagenicity. Extracts of copies without intentional print (that is, copies that come out of the machine blank) were sometimes weakly mutagenic, probably as a result of the presence of unintentional print. The mutagenic response with different tester strains suggests that the compound (or compounds) are causing frameshift mutations. Mutagenicity in the absence of the metabolic activation shows that the compound (or compounds) are either directly acting mutagens or are converted to mutagens by the bacterial enzymes present.

There were wide differences in the mutagenic responses of extracts from copies and toners from different copiers. Copies from a 5a machine (we have coded copier manufacturers by number and copier models by letter) gave extracts with a mutagenic response corresponding to about 10^3 revertants per page with TA98, whereas copies from a 3b machine gave a response of about 10^5 revertants per page. The other copies

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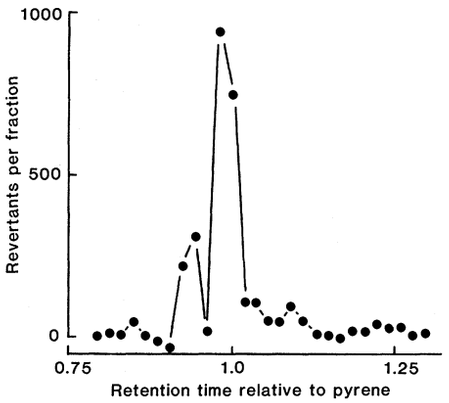

Fig. 1. Mutagenic response of fractions from a reverse-phase HPLC separation of the aromatic fraction of an extract from copies from a 4a machine. The sample, 50 μl containing activity corresponding to about 4000 revertants, was applied to a Spherisorb S5-ODS column (220 by 4.6 mm, internal diameter) with aqueous methanol as eluent, 2 ml per minute (gradient 38 percent methanol to 80 percent methanol for 10 minutes, then 80 percent methanol). Fractions (0.5 ml, 15 seconds) were collected and duplicate samples of 0.2 ml of each fraction were assayed for mutagenicity with TA98. The mutagenicity, expressed as net revertants above the spontaneous background per 0.5-ml fraction, is plotted against retention time relative to pyrene.

yielding mutagenic extracts gave responses between these values.

Subsequent studies of additional assays incubated anaerobically with TA98 (*2*) and the use of the nitroreductase-deficient strain TA98NR showed dissimilarities in the mutagenic patterns of extracts from copies and toners from different manufacturers. For example, the mutagenic patterns of 1a and 1b extracts appeared similar to those of 2a and 2b, but extracts from 3b and 4a had different patterns (Table 1).

The mutagenic pattern was similar for extracts of copies and toners from the same machine, an indication that toners are directly responsible for the mutagenicity in copies. If we assume that an average copy page contains approximately 25 mg of toner (*3*), the range of the mutagenic response given above corresponds to a variation between 40 and 4000 revertants per milligram of toner. Toners extracted with dimethyl sulfoxide (DMSO) (see legend to Table 1) gave lower mutagenic activity than that expected from the extraction of copies. This indicated that the extraction procedure used for the toners was incomplete. Soxhlet extraction of toners with acetone was also tried but shaking with DMSO gave a consistently higher response. No experiments were performed to evaluate the efficiency of the Soxhlet extraction of copies with acetone.

Fractionation of the mutagenic extracts from copies by a modification of the method described by Wynder and Hoffmann (*4*) confirmed the dissimilarities in the source of the mutagenic activity between the various products (Table 1). In copies from 1a, 1b, and 2a the mutagenic activity was mainly present in the neutral fraction and was eluted with benzene (aromatic fraction) from a silica column. In contrast, extracts of copies from 3b yielded no mutagenicity in the aromatic fraction; all activity was found in the acidic and basic fractions and in the neutral fractions eluted with diethyl ether and methanol (oxygenated fractions) from the silica column. In the case of 4a copies, mutagenicity was detected in all fractions except the neutral fraction eluted with cyclohexane (aliphatic fraction).

The aromatic fraction from the silica-column chromatography was further separated by reverse-phase high-performance liquid chromatography (HPLC) under conditions as given in Fig. 1. More than 80 percent of the mutagenicity in copy extracts from 1a, 1b, 2a, and 4a was found to elute close to pyrene (0.85 to 1.05 relative retention time). The retention time for reference compounds relative to pyrene were: 0.7 for 1-nitronaphthalene, 0.9 for 9-nitroanthracene, and 1.0 for 1-nitropyrene. The mutagenicity pattern suggested the presence of nitro groups whereas the chromatographic separations suggested the presence of tri- to pentacyclic aromatic structures.

Attempts were made to identify the mutagenic compound (or compounds) in the aromatic fraction from copies made on 1a machines by combined gas chromatography–mass spectrometry and by direct injection into the mass spectrometer (*5*). The concentration used for injection was the highest possible one practical (estimated to be of the order of 0.1 μg/μl) and was selected on the assumption that the mutagenic response was approximately 1000 revertants per microgram, which might be a reasonable average of the mutagenic response of inferred compounds (*2*). No differences were observed, however, between mutagenic samples and blank samples run at the same time, an indication that many compounds contributed to the mutagenicity, that the mutagenic response was much higher than assumed, or both.

Table 1. Mutagenicity of extracts of photocopies and toners from different copying machines. Three copies with normal print were extracted in a Soxhlet with acetone overnight; the acetone was then removed at reduced pressure and the residue dissolved in dimethyl sulfoxide (DMSO). Toners (1 g) were shaken with 20 ml of DMSO for 3 hours and then filtered to remove particles from the solution. A liquid toner (number 5) was tested directly. Mutagenicity was assayed with the *Salmonella* plate incorporation method with bacterial cultures grown overnight as described by Ames *et al.* (*1*). Anaerobic incubation means incubating the plates anaerobically during the first 16 hours of the 48-hour incubation at 37°C as described by Löfroth (*2*). Positive response was a linear or almost linear dose response and an elevation of revertants more than three times the spontaneous rate. (Mutagenic activity in revertants per page or per milligram of toner are not given since these values vary with amount of print per page and also from batch to batch of toner.) The original extract of copies was fractionated in acidic, basic, and neutral fractions. The acidic and basic fractions contained compounds extractable from diethyl ether by aqueous sulfuric acid and sodium hydroxide, respectively, and reextractable into ether after neutralization. The neutral fraction was further separated in four subfractions of increasing polarity on a silica column: an aliphatic, an aromatic, and two oxygenated fractions eluted by cyclohexane, benzene, diethyl ether, and methanol, respectively. N.D., not determined.

Photocopier		Mutagenicity detected with TA98		Mutagenic response of toners and copies relative to response in TA98			Fractions containing the major mutagenicity
Manufacturer	Model	Toner	Copies	Anaerobic incubation with TA98	TA98-NR	TA-1538	
1	a	+	+	< 0.5	~ 1	< 0.5	Aromatic
	b	+	+	< 0.5	~ 1	< 0.5	Aromatic
	c	−	−				
2	a	+	+	< 0.5	~ 1	< 0.5	Aromatic
	b	+	+	< 0.5	~ 1	< 0.5	N.D.
3	a	−	−				
	b	+	+	~ 1	< 0.2	~ 1	Oxygenated, acidic, basic
4	a	+	+	~ 0.5	~ 0.5	~ 0.5	Aromatic, oxygenated, basic, acidic
5	a	+	+	N.D.	N.D.	N.D.	N.D.
6	a	−	−				
7	a	−	−				

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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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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