

# Binding and Repair of 2-Acetylaminofluorene Adducts in Distinct Liver Cell Populations

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The study of the binding of the liver carcinogen, *N*-acetyl-2-aminofluorene, to the DNA of the target organ—as the probable initial step in the process of carcinogenesis—has shown that three modes of interaction occur. *N*-Acetyl-2-aminofluorene is covalently bound with the nitrogen to the carbon 8 of guanine (I) and with the 3-position to the free NH<sub>2</sub>-group of guanine (II). The third mode of interaction is formed by a covalent bond between the nitrogen of 2-aminofluorene and the carbon 8 of guanine (III). In this study the different modes of interaction were measured separately in stromal and parenchymal cells of the rat liver, after a single intraperitoneal dose. The DNA was isolated from nuclei that had been separated by 1g sedimentation. In parenchymal DNA the types of interaction I and III occur in the same amounts one day after application. In stromal cells the amount of interaction I is relatively small and interaction III predominates (ratio III:I = 5). The amount of interaction III in tetraploid hepatocytes (the largest cell population in the studied rats) per mg DNA is about two times higher than in the stromal cells. While the removal of the total amount of DNA-bound carcinogen takes place at the same rate in the two cell types, a difference in rate and efficiency of repair is observed for the different types of interaction. In tetraploid hepatocytes, interaction I is almost completely removed from the DNA 2 weeks after application, while interaction III diminishes to about 1/3 during the first week but the remaining part disappears very slowly. As shown in earlier studies, interaction II remains in the DNA at a constant level.

This paper reports on the work done at The Netherlands Cancer Institute in Amsterdam on the interaction of 2-aminofluorene and 2-acetylaminofluorene with DNA. The structure of these two compounds is shown in Figure 1, and both the amine and the acetyl derivative are well known as powerful liver carcinogens.

I would like to start by briefly summarizing the knowledge that has been accumulated during the past 15 years on the metabolism and the interaction of these compounds with DNA in the target organ. As a consequence of the metabolic activity in the rat liver, these compounds are interconverted by acetylating and deacetylating enzymes; besides these conversions a number of other derivatives are formed via liver metabolism (1). As far as we know now, two of these metabolites are reactive enough to interact directly with DNA *in vivo*, and the structures of these ultimate carcinogens are shown in

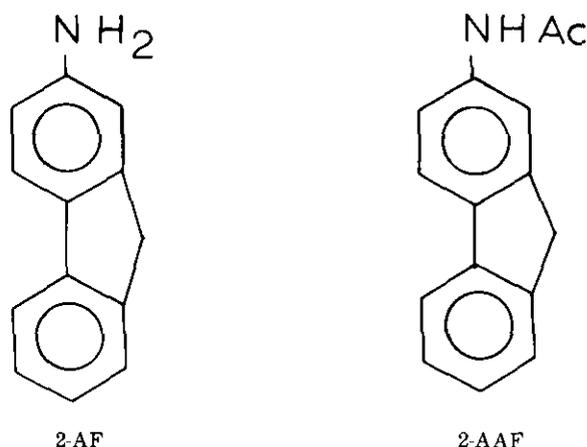


FIGURE 1. Structures of (2-AF) 2-aminofluorene and (2-AAF) 2-acetylaminofluorene.

Figure 2. 2-AAF-OR is formed by *N*-hydroxylation of 2-acetylaminofluorene followed by esterification, and it is probable that it is the sulfate ester (R = SO<sub>3</sub>) that plays the most important role in liver *in vivo*. The presence of this sulfate has, as a conse-

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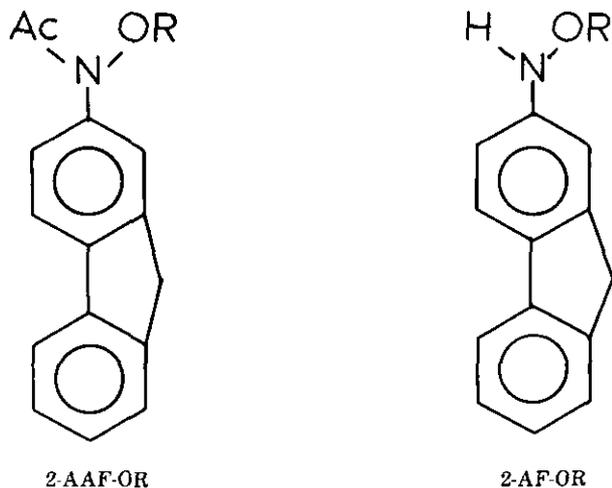


FIGURE 2. Ultimate carcinogens formed in the rat liver from AF and AAF. The nature of the leaving group (-OR) is not unambiguously known.

quence of its reactivity, not been demonstrated directly in the rat liver, but several indications have been obtained for its presence after application of the carcinogen (2). This compound has been prepared synthetically and decomposes rapidly in water but also reacts in aqueous solution with DNA at two different sites.

The first mode of interaction results in a covalent bond between the *ortho* position of the aromatic moiety of the carcinogen and the free amino group of guanine in DNA; in addition to this reaction, the position 8 of guanine is attacked by the nitrogen of the carcinogen (3). Figure 3 shows the structures of the interactions, and compounds I and II can be isolated from the reaction mixture after acid hydrolysis of the DNA. These two compounds can be isolated from the *in vitro* reaction as well as from rat liver DNA after treatment of the animal with the carcinogen. Compound III in Figure 3 is also detected in liver DNA hydrolyzates (4) after carcinogen application and can be obtained *in vitro* by deacetylation of compound II (5). This third mode of interaction *in vivo* probably occurs as a consequence of the interaction of the ultimate carcinogen 2-AF-OR shown in Figure 2. There are indications that *in vivo* it is the O-acetate (R = acetyl) that is responsible for this particular DNA interaction, but the glucuronide may also play a role as leaving group (6). Thus, interactions I and II occur simultaneously via one metabolic pathway while interaction III most likely occurs independently via another. An experiment done with 3-month-old male rats of the strain R-Amsterdam to determine the amounts of the three modes of interaction shows, one day after a single intraperitoneal dose of the tritiated 2-ac-

tylaminofluorene, a ratio of 1:4:10 = I:II:III (Fig. 3). This ratio however appeared to depend on the sex (7), the strain (8), the period between application and sacrifice of the animal (9) and probably on the age.

Figure 4 demonstrates the strain dependence of the ratio between the interactions II and III in the rat liver one day after application. These chromatograms show the amounts of GuAF and GuAAF measured by the amounts of radioactivity that cochromatographed with the synthetic marker compounds that had been added to the acid hydrolyzates of the liver DNA. Where the amount of the acetylated product is about the same in both strains, the deacetylated bound form is about four

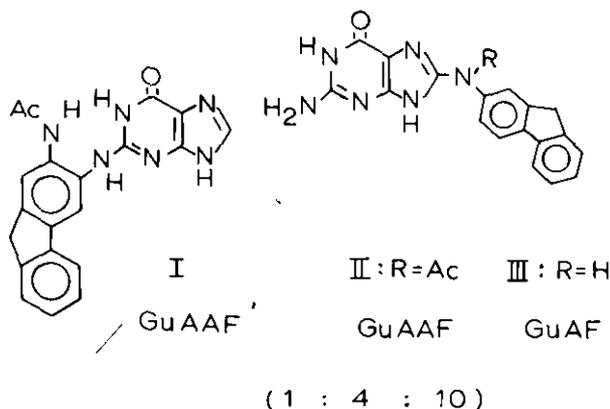


FIGURE 3. Interaction products of AF and AAF isolated from the acid hydrolyzate of rat liver DNA after application of AAF. The ratio 1:4:10 is observed in 3-month-old male rats of the strain R-Amsterdam, 1 day after a single IP dose of 3 mg/kg body weight.

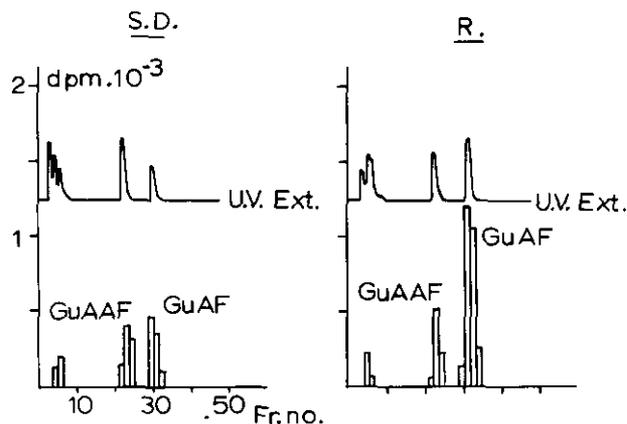


FIGURE 4. Chromatograms of acid liver DNA hydrolyzates, 1 day after a single IP dose of [<sup>3</sup>H]AAF. The marker compounds GuAAF and GuAF were added to the hydrolyzates. The difference between the two rat strains is demonstrated by the amounts of radioactivity that cochromatograph with the marker compounds.

times lower in the Sprague-Dawley males, and obviously this is caused by strain-dependent differences in hepatic metabolism in the liver of the different strains. These hydrolyzates were obtained by treatment of the DNA with trifluoroacetic acid. This procedure has the advantage that hydrolysis proceeds rapidly and quantitatively for both the carbon 8-substituted adducts (II and III). The N<sup>2</sup>-substituted adduct, however, decomposes under these conditions (10), which is the reason these chromatograms give no information about interaction I. This latter mode of interaction has been studied earlier in our laboratory and was found to be persistently present in the liver (9).

In this present study we were particularly interested in the rate of disappearance of products from the guanine carbon 8 interactions in the liver. Figure 5 shows the decrease from the liver DNA of the two guanine carbon-8-substituted adducts in 3-month-old rats of the strain R-Amsterdam after a single IP dose. The vertical lines give the coefficient of variation of the amounts of radioactivity that co-chromatographed with the synthetic markers. Four animals were used for every point. The shape of this curve strongly suggests that the amounts of both interactions decrease to a constant value (8) and in one experiment we observed that after a period of 10 weeks both the adducts were still present in the liver DNA in the same amounts as after 2 weeks.

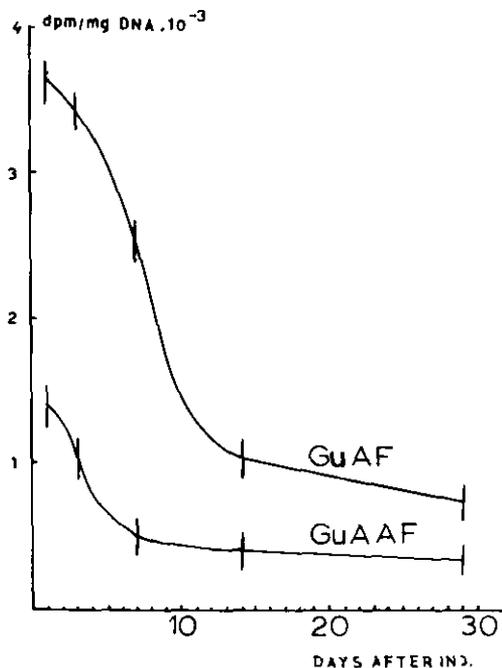


FIGURE 5. Decrease of the amounts of 8-guanine substituted adducts in rat liver DNA (males, strain R-Amsterdam) after a single IP dose of [<sup>3</sup>H]AAF.

As a consequence of these results, the question arose whether this persistently bound fraction was located in distinct cell populations in the liver or if it was spread out randomly over the whole liver. In order to answer this question, we decided to use a technique for the separation of nuclei from different cell populations of the liver by velocity sedimentation at unit gravity (11). Figure 6 shows the sedimentation chamber that we used. A nuclear suspension of 100 mL containing about 10<sup>9</sup> nuclei, obtained from a liver homogenate was layered on the top of a sucrose gradient ranging from 5 to 30% over an 8-cm distance. The nuclei were allowed to sediment overnight at 4°C. Figure 6 shows schematically the separation that is obtained under these conditions.

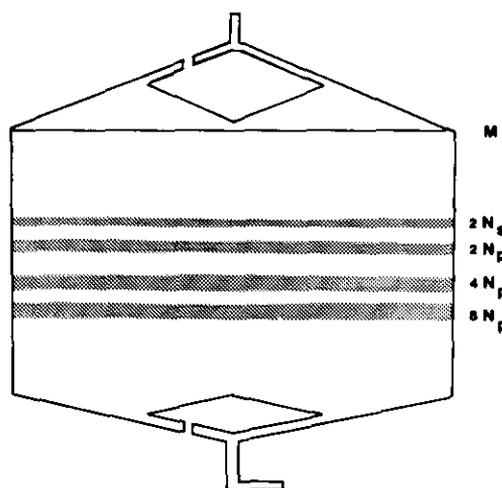


FIGURE 6. Separation of stromal and parenchymal nuclei in a separation chamber on a sucrose gradient at 1g.

By slowly introducing dense cushion liquid into the bottom of the chamber, the contents of the chamber is fractionated via the top cone within 20 min. In the isolated fractions the number of nuclei was counted in a Coulter counter and ploidy was measured by pulse cytophotometry or by Coulter counter size analysis. A separation of diploid stromal nuclei (mainly from Von Kupffer cells and endothelial cells), diploid parenchymal, tetraploid parenchymal and octaploid parenchymal nuclei was obtained. With increasing age of the animal, the amount of the diploid parenchymal cells in the liver diminishes in favor of the number of tetraploid and octaploid cells (12).

Figure 7 shows a diagram of the number of nuclei plotted against the fraction number of the fractions obtained from the separation chamber. In this case a 3-month-old male rat of the strain R-Amsterdam was used. Though this diagram shows that the separation of the different nuclei was not complete, it

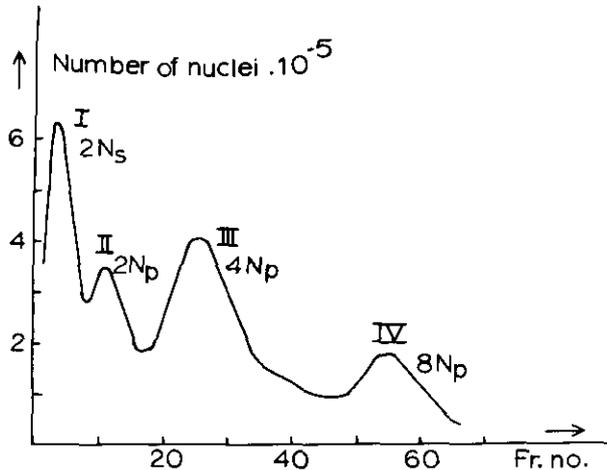


FIGURE 7. Diagram of the number of nuclei plotted against the fraction number of the fractions obtained from the top of the separation chamber shown in Figure 6.

appeared to be sufficient to obtain more insight into the differences in binding of aminofluorene and acetylaminofluorene to DNA in these distinct cell classes of the rat liver.

The scheme used for these experiments was (1) injection of  $^3\text{H}$ -AAF (900 mCi/mmol); (2) isolation of nuclei; (3) separation of nuclei at 1g; (4) DNA isolation; (5) DNA hydrolysis with TFA; (6) HPLC of the hydrolyzates with the marker compounds. R-Amsterdam rats of about 3 months were injected with highly labeled acetylaminofluorene, tritiated in the aromatic moiety at a dose level of 2 mg/kg body weight, and the animals were sacrificed 2, 7 and 14 days after application, respectively. Nuclei were isolated from the liver homogenate and separated at unit gravity. From the pooled fractions the DNA was isolated by sodium dodecyl sulfate lysis followed by hydroxyapatite chromatography. The DNA was purified further by precipitation of its cetavlon salt and analyzed by hydrolysis in trifluoroacetic acid and chromatography of the hydrolyzate by HPLC on a reversed phase column with a propanol/water gradient.

Figure 8 shows the chromatograms of two hydrolyzates. The left one is from tetraploid hepatocytes and the right one is from stromal diploid nuclei. The animals were sacrificed 2 days after carcinogen application and about 200  $\mu\text{g}$  DNA was hydrolyzed and chromatographed. The marker compounds were added to the hydrolyzate, and these chromatograms show that the amount of deacetylated bound material is about the same in the tetraploid parenchymal cells and in the non-parenchymal liver cells. The amount of acetylated material, however, is very small in the stromal cells as compared with the tetraploid hepatocytes. The chromatograms of the dip-

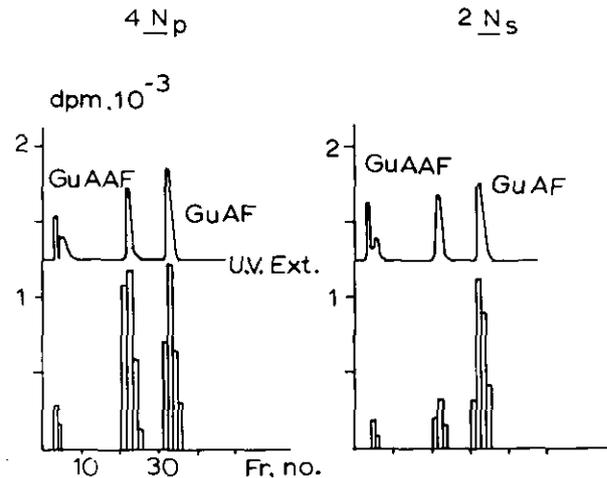


FIGURE 8. Chromatograms of the trifluoroacetic acid hydrolyzates of 200  $\mu\text{g}$  DNA, obtained from the fractions of the stromal nuclei and the tetraploid hepatocyte nuclei 2 days after a single IP dose of  $^3\text{H}$ -AAF.

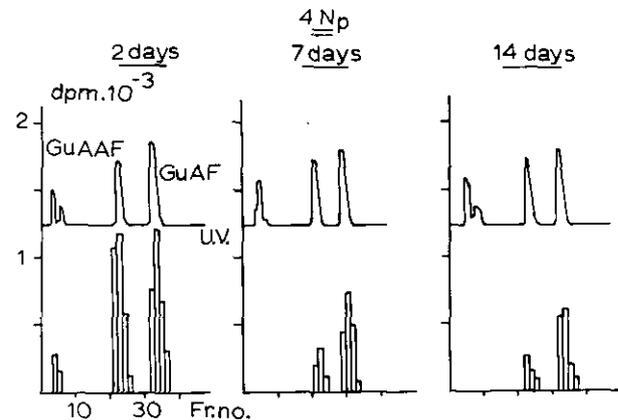


FIGURE 9. Chromatograms of 200  $\mu\text{g}$  DNA of the tetraploid hepatocytes at different stages after application.

loid hepatocytes show a picture that lies between the two chromatograms shown in Figure 8. The reproducibility of the analysis of the diploid hepatocyte nuclei was rather poor and probably this is caused by the overlap of peaks I and II in the diagram of the nuclei separation (Fig. 7). When we assume that in the interaction of the acetylated bound aminofluorene the sulfate transferase plays an essential role, then we may conclude from this figure that this enzymatic activity in the nonparenchymal cells is much lower than in the hepatocytes.

Figure 9 shows what happens with the two modes of interaction during the first 2 weeks after application. These three chromatograms are from the hydrolyzates of tetraploid hepatocytes 2, 7 and 14 days after application of the tritiated carcinogen. We observe that both modifications disappear from

the DNA, but at a different rate. Obviously, the repair mechanism in the tetraploid hepatocytes has a different efficiency for the different modes of interaction. When we compare these results with earlier studies of AF- and AAF-modified DNA with respect to their sensitivity towards nuclease  $S_1$  (13) and with studies on the conformational changes caused by these modifications (14), then we can conclude that a correlation exists between the amount of disturbance of the DNA helix and the rate of removal of the modification *in vivo*.

Table 1. Amounts of DNA interaction products in hepatocytes and stromal cells in the rat liver (3-month-old male rats, strain R-Amsterdam) after a single application of [ $^3$ H]AAF (900 mCi/mole) at a dose level of 2 mg/kg body weight.

	Time after dose, days	Activity, dpm/mg	
		GuAAF	GuAF
Hepatocytes (III and IV)	2	14000	16000
	7	4500	11500
	14	2000	10000
Stromal cells (I)	2	2300	13000
	7	<1000	9500
	14	—	8000

Table 1 shows the quantitative values of the two different modes of interaction in the hepatocytes and nonhepatocytes in the liver and gives an impression of the rate of disappearance from the DNA. For the tetraploid hepatocytes and the octaploid hepatocytes, we observed the same values. The values of the diploid hepatocytes are omitted in this figure for two reasons: first, the amount of DNA that we isolated from the diploid hepatocyte fractions was, in most cases, too low for a reliable analysis and second, the diploid hepatocyte nuclei in most cases, were contaminated with nuclei from stromal cells to a large extent, which is in contrast with the stromal nuclei fraction, which appeared to contain diploid hepatocyte nuclei to a very small extent.

With respect to the amount of deacetylated bound materials in both cell classes, these data show the same trend as we had observed for the total liver DNA: after a rapid decrease of the interaction product during the first week, the rate of disappearance decreases, and this holds for the stromal cells as well as for the hepatocytes. For the acetylated-bound material in the hepatocytes, we observe the same effect, with this difference that the repair appears to be more complete.

The amount of acetylated-bound carcinogen in the DNA of the stromal cells after 1 week could not be analyzed reliably as a consequence of the low level of the interaction and the relatively small amount

of DNA that we could isolate from this fraction. In conclusion we can state that, although there is a difference in interaction in the liver between the different cell populations, a significant difference in repair between the hepatocytes and the nonhepatic cells in the liver seems not to exist. In order to make a further differentiation in the stromal cell fraction between the von Kupffer cells and the endothelial cells, we shall have to use other separation methods (elutriation techniques) (15), and for a more precise determination of the half-life time of the different modes of interaction in the distinct liver cells, we hope that we can use immunological methods (16) for the quantitative analysis.

## REFERENCES

- Kriek, E., and Westra, J. G. Metabolic activation of aromatic amines and amides and interaction with nucleic acids. In: Chemical Carcinogens and DNA (P. L. Gover, Ed.), Vol. II, CRC Press, Boca Raton, FL, 1979, pp. 1-28.
- Meerman, J. H. N., Van Doorn, A. B. D., and Mulder, G. J. Inhibition of sulfate conjugation of *N*-hydroxy-2-acetylaminofluorene in isolated perfused rat liver and in the rat *in vivo* by pentachlorophenol and low sulfate. *Cancer Res.* 40: 3772-3779 (1980).
- Westra, J. G., Kriek, E., and Hittenhausen, H. Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA *in vivo*. *Chem.-Biol. Interact.* 1: 287-303 (1976).
- Beland, F. A., Dooley, K. L., and Casciano, D. A. Rapid isolation of carcinogen bound DNA and RNA by hydroxyapatite chromatography. *J. Chromatogr.* 174: 177-186 (1979).
- Kriek, E., Miller, J. A., Juhl, U., and Miller, E. C. 8-(*N*-2-Fluorenylacetyl-amido)-guanosine, an arylamidation reaction product of guanosine and the carcinogen *N*-acetoxy-*N*-2-fluorenylacetyl-amide in neutral solution. *Biochemistry* 6: 177 (1967).
- Beland, F. A., Allaben, W. T., and Evans, F. E. Acetyltransferase mediated binding of *N*-hydroxyarylamides to nucleic acids. *Cancer Res.* 40: 834-840 (1980).
- Kriek, E. On the mechanism of action of carcinogenic aromatic amines. I. Binding of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene to rat liver nucleic acids *in vivo*. *Chem.-Biol. Interact.* 1: 3-17 (1969).
- Visser, A., and Westra, J. G. Partial persistency of 2-aminofluorene and *N*-acetyl-2-aminofluorene in rat liver DNA. *Carcinogenesis* 2: 737-740 (1981).
- Kriek, E. Persistent binding of a new reaction product of the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene with guanine in rat liver DNA *in vivo*. *Cancer Res.* 32: 2042-2048 (1972).
- Westra, J. G., and Visser, A. Quantitative analysis of *N*-(guanin-8-yl)-*N*-acetyl-2-aminofluorene and *N*-(guanin-8-yl)-2-aminofluorene in modified DNA by hydrolysis in trifluoroacetic acid and high pressure liquid chromatography. *Cancer Letters* 8: 155-162 (1979).
- Tulp, A., Welagen, J. J. M. N., and Westra, J. G. Binding of the chemical carcinogen *N*-hydroxyacetylaminofluorene to ploidy classes of rat liver nuclei as separated by velocity sedimentation at unit gravity. *Chem.-Biol. Interact.* 23: 293-303 (1978).
- Nadal, C., and Zajdela, F. Polyploidie somatique dans le

- foie de rat. *Exptl. Cell Res.* 42: 99-116 (1966).
13. Fuchs, R. P. P. *In vitro* recognition of carcinogen induced local denaturation sites in native DNA by S<sub>1</sub> endonuclease from *Aspergillus oryzae*. *Nature* 257: 151-152 (1975).
  14. Santella, R. M., Kriek, E., and Grunberger, D. Circular dichroism and proton magnetic resonance studies of dApdG modified with 2-aminofluorene and 2-acetylaminofluorene. *Carcinogenesis* 1: 897-902 (1980).
  15. McEwen, C. R., Stallard, R. W., and Juhas, E. T. Separation of biological particles by centrifugal elutriation. *Anal. Biochem.* 23: 369-377 (1968).
  16. Van Der Laken, C. J., Hagenars, A. M., Hermsen, G., Kriek, E., Kuipers, A. J., Nagel, J., Scherer, E., and Welling, M. Measurement of O<sup>6</sup>-ethyldeoxyguanosine and N-(deoxyguanosin-8-yl)-N-acetyl-2-aminofluorene in DNA by high-sensitive enzyme immuno-assays. *Carcinogenesis*, in press.