# CARCINOGENIC NON-AMINOAZO DYE 1-PHENYLAZO-2-HYDROXYNAPHTHALENE (SUDAN I) IS OXIDIZED BY RAT LIVER MICROSOMAL CYTOCHROME P-450 TO METABOLITES BINDING TO MACROMOLECULES (NUCLEIC ACIDS AND PROTEINS)

Marie STIBOROVÁ[a] and Pavel ANZENBACHER[b]

[a] *Department of Biochemistry, Charles University, 128 40 Prague 2*
[b] *Institute of Physiology, Czechoslovak Academy of Sciences, 142 20 Prague 4*

Received May 21, 1991
Accepted November 8, 1991

Carcinogenic non-aminoazo dye 1-phenylazo-2-hydroxynaphthalene (Sudan I) is oxidized by microsomal cytochromes P-450 to reactive metabolite(s) binding to macromolecules (nucleic acids, proteins) in vitro. The extent of binding to macromolecules proceeded in the order: protein > rRNA > tRNA > DNA. The pattern of products formed from Sudan I and binding of the reactive metabolites of this compound to macromolecules are dependent on the concentrations of Sudan I, NADPH and on the duration of the incubation. The participation of the adducts formed with macromolecules in the initiation of chemical carcinogenesis is discussed.

The non-aminoazo compound Sudan I (1-phenylazo-2-hydroxynaphthalene) is a hepatocarcinogen and a urinary bladder carcinogen in mice and rabbits[1,2]. It was found previously that microsomal enzymes of rat liver metabolized Sudan I mainly to C-hydroxy derivatives and formation of the benzenediazonium ion was also detected. The binding of Sudan I activated by the microsomal system, to DNA in vitro was also ascertained. The benzenediazonium ion has been supposed to be the ultimate carcinogen responsible for the formation of DNA adducts[3,4].

The present paper continues our studies of Sudan I metabolism by microsomal enzymes and it demonstrates the binding of the activated Sudan I not only to DNA, but also to RNA and microsomal proteins.

## EXPERIMENTAL

### Chemicals

Sudan I (1-phenylazo-2-hydroxynaphthalene) (British Drug Houses), calf thymus DNA and NADPH (Boehringer Mannheim, F. R. G.), 5,6-benzoflavone (Sigma, U. S. A.) and other chemicals (Lachema Brno, Czechoslovakia) were of analytical grade. Rat liver tRNA was prepared as described by Rogg et al.[5].

The labelled 1-([U-$^{14}$C]phenylazo)-2-hydroxynaphthalene ($^{14}$C-Sudan I, 20 MBq mmol$^{-1}$) was synthesized as described in an earlier paper[6] from [U-$^{14}$C]aniline (The Radiochemical Centre, Amersham,

England) and β-naphthol, and purified by column chromatography on basic alumina and preparative thin-layer chromatography (TLC) on silica gel. The labelled compound was stored in a methanol solution at −5 °C.

Subcellular Preparations

Male Sprague–Dawley rats (about 100 – 150 g) were injected i.p. with 0.4% 5,6-benzoflavone dissolved in maize oil (60 mg $kg^{-1}$ body weight) once a day for 3 consecutive days. Animals were starved for 16 – 18 h prior killing, and liver microsomes were prepared as described by Kimura et al.[7] and stored at −70 °C.

Incubations

Unless stated otherwise, to 1.4 ml of the incubation mixture containing 50mM potassium phosphate buffer (pH 7.7) and 2mM NADPH, 3.5 mg of microsomal proteins, 100 μl of 3mM $^{14}$C-Sudan I dissolved in methanol and 2 mg of DNA or tRNA were added. The mixtures were extracted after incubation (37 °C, 120 min) with ethyl acetate (2 × 2 ml). The amounts of reaction products and of the residual $^{14}$C-Sudan I were determined in ethyl acetate extracts as described previously[3,8]. Microsomes were reisolated from the residual aqueous phases by centrifugation (60 min at 105 000 *g*, 4 °C) and both, pellets and supernatants were collected separately. The extraction of Sudan I metabolites by ethyl acetate does not change those properties of microsomal proteins which are essential for their reisolation by centrifugation[3,4]. Pellets of microsomes were then resuspended in one ml of 50mM Tris-HCl buffer, pH 7.5. One milliliter of 80% phenol and 1 ml of chloroform were then added to the resuspended microsomes, mixtures were shaken vigorously and centrifuged (10 min at 5 000 *g*, 20 °C). The aqueous phases were transferred into other test tubes and the phenol-chloroform extraction procedure was repeated. Small volume (100 μl) of 3M sodium acetate was added to the resulting aqueous phases. RNA (mainly rRNA), which is always present in the microsomal fractions containing ribosomes[7], was then precipitated from aqueous phases by ethanol (5 ml). In experiments with added tRNA or DNA to the incubation mixtures, similar procedure was used. However, one milliliter of 80% phenol and 1 ml of chloroform were added to supernatants obtained after reisolation of microsomes containing tRNA or DNA and the mixtures were shaken vigorously and centrifuged as described above. Small volume (100 μl) of 3M sodium acetate was added to the aqueous phases. Then, tRNA or DNA was precipitated by ethanol (5 ml).

Precipitates of nucleic acids were washed with ethanol, ethanol–diethyl ether (1 : 1, v/v), diethyl ether (2 ×) and dried under a stream of nitrogen. DNA, tRNA or rRNA were dissolved separately in 0.5 ml of distilled water and dialyzed against distilled water for the removal of the impurities (24 h). The $^{14}$C radioactivity of nucleic acids was determined in 100 – 400 μl aliquots by means of liquid scintillation counting (Packard Tri-Carb 2000 CA). The content of nucleic acids was measured spectrophotometrically[5,9]. Microsomal proteins were then recovered from the phenol-chloroform phases. Acetone (5 ml) was added to the residual phenol-chloroform layer containing denatured microsomal proteins. Precipitated proteins were centrifuged and washed by acetone (2 ×), ethanol (2 ×), ethanol–diethyl ether (1 : 1), diethyl ether (2 ×) and dried under a stream of nitrogen. Precipitates were dissolved in 1 ml of 0.1M NaOH (at 65 °C) and the $^{14}$C radioactivity of proteins was determined in 100 μl aliquots by means of liquid scintillation counting. The content of protein was measured by the method of Bradford[10] with bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

Microsomal enzymes of rat livers containing cytochrome P-450 activate Sudan I to form metabolites modifying nucleic acids and proteins in vitro. The extent of binding to microsomal proteins was the highest and proceeded in the order: protein > rRNA > tRNA > DNA in incubation mixtures containing only microsomes (with the rRNA

present due to presence of ribosomes) or microsomes and DNA or microsomes and tRNA. In the absence of microsomal enzymes, no binding was observed (Table I). The binding to macromolecules is dependent on the concentration of NADPH in incubation mixtures (Table II).

TABLE I
Binding of $^{14}$C-Sudan I activated by microsomes to macromolecules in vitro. The values given are averages of three parallel experiments together with standard deviations. For other conditions see Experimental

| Reaction mixture | Binding to[a] | | | |
|---|---|---|---|---|
| | protein | DNA | tRNA | rRNA |
| Complete | 13.90 ± 0.90 | 0.40 ± 0.02 | 0.53 ± 0.02 | 1.52 ± 0.05 |
| Without microsomes | 0 | 0 | 0 | 0 |
| Without NADPH | 0.10 ± 0.001 | 0.01 ± 0.001 | 0.09 ± 0.003 | 0.08 ± 0.002 |

[a] In nmol mg$^{-1}$.

TABLE II
Effect of NADPH on the binding of $^{14}$C-Sudan I activated by microsomes to macromolecules. The values given are averages of three parallel experiments and standard deviations. For other conditions see Experimental

| NADPH mM | Binding to[a] | | | |
|---|---|---|---|---|
| | protein | DNA | tRNA | rRNA |
| 0 | 0.10 ± 0.001 | 0.01 ± 0.001 | 0.09 ± 0.003 | 0.08 ± 0.002 |
| 0.5 | 3.50 ± 0.01 | 0.15 ± 0.009 | 0.25 ± 0.009 | 0.70 ± 0.002 |
| 1.0 | 8.23 ± 0.20 | 0.30 ± 0.01 | 0.40 ± 0.01 | 1.10 ± 0.03 |
| 1.5 | 13.00 ± 0.90 | 0.38 ± 0.01 | 0.50 ± 0.02 | 1.45 ± 0.05 |
| 2.0 | 13.90 ± 0.90 | 0.40 ± 0.02 | 0.53 ± 0.02 | 1.52 ± 0.05 |

[a] In nmol mg$^{-1}$.

Furthermore, binding of the activated $^{14}$C-Sudan I metabolites to macromolecules is dependent on the length of incubation (Table III) and on the amount of $^{14}$C-Sudan I present (Table IV). The highest level of binding is reached after 120 min.

Increasing concentrations of $^{14}$C-Sudan I (up to 0.1 mmol $l^{-1}$) enhances the binding to macromolecules, however, under concentrations of $^{14}$C-Sudan I higher than 0.1 mmol $l^{-1}$ the binding of $^{14}$C-Sudan I metabolites is decreased. These results obtained seem to be rather surprising. The metabolism of $^{14}$C-Sudan I by microsomal enzymes was, hence, studied in more detail. Different concentrations of $^{14}$C-Sudan I in the incubation mixture at constant concentrations of microsomal enzymes and NADPH lead to the formation of different pattern of products. At the concentrations of $^{14}$C-Sudan I higher than 0.15 mmol $l^{-1}$, major part of products formed are C-hydroxy derivatives of Sudan I: 1-(4′-hydroxyphenylazo)-2-hydroxynaphthalene (*VI*) and 1-phenylazo-2,6-dihydroxynaphthalene (*V*). Another product, which was detected as a benzenediazonium ion (or the compound raised from this ion-product *I*)[3,11] is, however, formed as

TABLE III

Dependence of the binding of $^{14}$C-Sudan I metabolites to macromolecules after activation with the microsomal system on incubation time. The means and standard deviations were obtained from triplicate determinations. For other conditions see Experimental

| Time min | Binding to[a] | | | |
|---|---|---|---|---|
| | protein | DNA | tRNA | rRNA |
| 0 | 0.01 ± 0.001 | 0 | 0 | 0 |
| 10 | 3.80 ± 0.01 | 0.10 ± 0.008 | 0.18 ± 0.005 | 0.40 ± 0.001 |
| 20 | 6.25 ± 0.20 | 0.14 ± 0.009 | 0.25 ± 0.008 | 0.65 ± 0.02 |
| 40 | 8.80 ± 0.20 | 0.20 ± 0.01 | 0.38 ± 0.01 | 1.10 ± 0.03 |
| 60 | 10.80 ± 0.80 | 0.28 ± 0.01 | 0.48 ± 0.02 | 1.45 ± 0.05 |
| 100 | 13.89 ± 0.90 | 0.36 ± 0.02 | 0.53 ± 0.02 | 1.50 ± 0.05 |
| 120 | 13.90 ± 0.90 | 0.40 ± 0.02 | 0.53 ± 0.02 | 1.52 ± 0.05 |

[a] In nmol $mg^{-1}$.

the minor portion of products. On the other hand, this product is formed as one of the major products when concentrations of $^{14}$C-Sudan I are lower then 0.1 mmol $l^{-1}$. Two unknown products, *II* and *IV*, together with 1-(4'-hydroxyphenylazo)-2,6-dihydroxynaphthalene (*III*) and Sudan I (*VII*) were also detected (Table V). As benzenediazonium ion was determined previously as the ultimate carcinogenic metabolite of Sudan I which binds to DNA (ref.[4]), results presented in this paper confirmed the earlier determinations. If the benzenediazonium ion is formed as one of the predominant products of Sudan I metabolism by microsomal enzymes, the labelling of DNA (RNA or protein) by $^{14}$C-Sudan I metabolites is very effective, too.

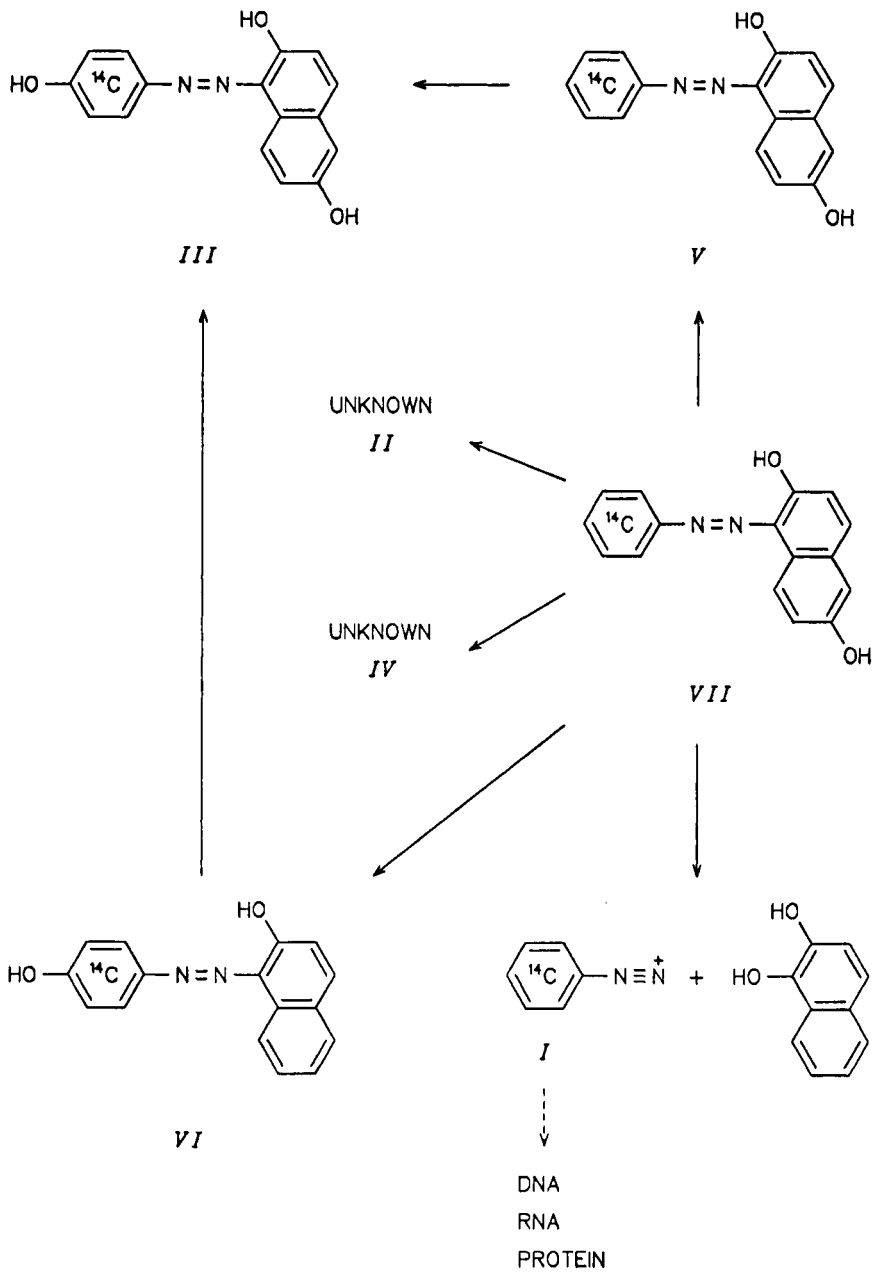
The formation of benzenediazonium ion from Sudan I by microsomal enzymes is strictly time-dependent, being formed predominantly at the beginning of incubation (Table VI). With the increasing duration of incubations, no further formation of this product is observed, the Sudan I metabolism is changed with respect to production of C-hydroxy derivatives of Sudan I.

It could be suggested from these findings that reactions leading to oxidative splitting of azo group of Sudan I (in other words, to the formation of the benzenediazonium ion) are the first reactions of Sudan I metabolism, carbon atoms of aromatic rings being the second targets for oxidation (hydroxylation) (Scheme 1). The reaction leading to the

TABLE IV
*Effect of concentration of $^{14}$C-Sudan I on the binding of metabolites to macromolecules.* The means and standard deviations were obtained from triplicate determinations. For other conditions see Experimental

| $^{14}$C-Sudan I mM | Binding to[a] | | | |
|---|---|---|---|---|
| | protein | DNA | tRNA | rRNA |
| 0 | 0 | 0 | 0 | 0 |
| 0.05 | 12.83 ± 0.80 | 0.41 ± 0.02 | 0.61 ± 0.02 | 1.38 ± 0.04 |
| 0.10 | 17.31 ± 0.90 | 0.48 ± 0.03 | 0.66 ± 0.03 | 1.53 ± 0.06 |
| 0.20 | 13.90 ± 0.90 | 0.40 ± 0.02 | 0.53 ± 0.02 | 1.52 ± 0.05 |
| 0.30 | 12.80 ± 0.90 | 0.36 ± 0.02 | 0.27 ± 0.02 | 0.69 ± 0.02 |
| 0.40 | 12.02 ± 0.80 | 0.36 ± 0.02 | 0.24 ± 0.02 | 0.48 ± 0.02 |

[a] In nmol $mg^{-1}$.




SCHEME 1
Products of oxidation of $^{14}$C-Sudan I by the microsomal system

formation of benzenediazonium ion is apparently rapid (as it possibly may be represented at the beginning as a pseudomonomolecular reaction, i.e. cleavage of the molecule); the mechanism of reactions leading to C-hydroxylated products may be more complicated (the insertion of oxygen atom). Hence, when Sudan I is present in the incubation mixture at low concentrations and the whole amount of Sudan I is converted rapidly, this compound could be oxidized mainly to benzenediazonium ion which is the reactive ultimate carcinogen.

Formation of different Sudan I products in the dependence on the length of incubation (or on the Sudan I concentrations) could also be explained in another manner. Microsomal enzymes (cytochromes P-450), which were used in the experiments were induced by 5,6-benzoflavone. That means that the microsomal preparations were rich in

TABLE V
Effect of of $^{14}$C-Sudan I concentration on the proportion of products formed from this compound by the microsomal system expressed in relative radioselectivity[a] (%). Incubation mixture without addition of nucleic acids. For other conditions see Experimental

| Product[b] | Concentration, mmol $l^{-1}$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.05 | 0.10 | 0.16 | 0.20 | 0.30 | 0.40 | 0.50 |
| *I* | 78.7 ± 0.7 | 38.5 ± 0.3 | 16.5 ± 0.1 | 12.5 ± 0.1 | 4.1 ± 0.04 | 3.3 ± 0.03 | 2.3 ± 0.02 |
| *II* | 3.3 ± 0.03 | 11.1 ± 0.1 | 6.6 ± 0.6 | 5.3 ± 0.05 | 1.4 ± 0.1 | 1.1 ± 0.1 | 1.2 ± 0.1 |
| *III* | 5.5 ± 0.5 | 27.2 ± 0.3 | 19.1 ± 0.2 | 13.3 ± 0.1 | 3.2 ± 0.03 | 2.3 ± 0.02 | 2.0 ± 0.02 |
| *IV* | 2.4 ± 0.2 | 0.9 ± 0.1 | 0.6 ± 0.05 | 0.3 ± 0.03 | 0.2 ± 0.02 | 0.2 ± 0.01 | 0.2 ± 0.02 |
| *V* | 2.0 ± 0.1 | 2.4 ± 0.2 | 17.0 ± 0.9 | 16.3 ± 0.7 | 21.3 ± 1.2 | 18.7 ± 0.3 | 16.4 ± 1.0 |
| *VI* | 4.2 ± 0.01 | 16.8 ± 1.2 | 36.5 ± 0.66 | 41.4 ± 0.8 | 34.1 ± 0.9 | 26.7 ± 2.1 | 24.4 ± 1.9 |
| *VII* | 3.9 ± 0.5 | 3.1 ± 0.09 | 3.7 ± 0.9 | 10.9 ± 1.0 | 35.7 ± 3.1 | 47.4 ± 1.9 | 53.5 ± 0.8 |

[a] Mean and standard deviation; total radioactivity was 217.57, 535.64, 1 017.41, 1 210.34, 2 568.01, 3 146.87 and 4 985.32 Bq for 0.05, 0.10, 0.16, 0.20, 0.30, 0.40 and 0.50 mM $^{14}$C-Sudan I, respectively. [b] Compounds separated by TLC (diethyl ether–petroleum ether 3 : 1) after extraction by ethyl acetate (see Experimental).

cytochrome P-448 (ref.[7]). The presence of other isoenzymes of cytochrome P-450 (in minority) could not, however, be excluded. Cytochrome P-448 present in the microsomes in the majority may prefer mainly such an orientation of substrates which is more appropriate for the oxidative splitting of Sudan I than for C-hydroxylation. On the other hand, minor isoenzymes of cytochrome P-450 can prefer C-hydroxylation of Sudan I. The products of reactions catalyzed by these minor isoenzymes (because of their low concentrations) may contribute to the detection of C-hydroxy derivatives, but only after a prolonged time of incubation (or at higher Sudan I concentrations).

Interaction of chemical carcinogens or their metabolites with macromolecules in the target cell seems to be an essential event in chemical carcinogenesis in the case of the so called genotoxic carcinogens[12].

TABLE VI

The time-dependence $^{14}$C-Sudan I conversion by the microsomal system expressed in relative radioactivity[a] (%). Incubation mixture containing 0.16mM $^{14}$C-Sudan I and without addition of nucleic acids. For other conditions see Experimental

| Product[b] | Incubation time, min | | | | |
|---|---|---|---|---|---|
| | 0 | 15 | 30 | 60 | 120 |
| *I* | 0.2 ± 0.01 | 37.5 ± 1.8 | 28.7 ± 0.9 | 16.4 ± 0.8 | 16.5 ± 0.8 |
| *II* | 0 | 6.1 ± 0.5 | 9.8 ± 0.9 | 11.7 ± 0.7 | 6.6 ± 0.4 |
| *III* | 0 | 10.0 ± 1.2 | 10.9 ± 1.1 | 20.0 ± 0.7 | 19.1 ± 0.7 |
| *IV* | 0 | 1.4 ± 0.1 | 1.3 ± 0.1 | 1.2 ± 0.11 | 0.6 ± 0.04 |
| *V* | 0 | 9.5 ± 0.7 | 13.1 ± 0.8 | 16.9 ± 1.0 | 17.0 ± 0.9 |
| *VI* | 0 | 26.3 ± 1.0 | 30.3 ± 1.8 | 30.2 ± 1.3 | 36.5 ± 0.9 |
| *VII* | 99.8 ± 2.5 | 9.2 ± 0.9 | 5.9 ± 0.4 | 3.6 ± 0.2 | 3.7 ± 0.2 |

[a] Mean and standard deviation: total radioactivity was 1 025.53, 1 047.06, 999.88, 1 001.48 and 1 017.41 Bq for 0, 15, 30, 60 and 120 min, respectively. [b] Compounds separated by TLC (diethyl ether–petroleum ether 3 : 1) after extraction by ethyl acetate (see Experimental).

The in vivo binding of $^{14}$C-Sudan I metabolites to nucleic acids of rat livers was detected previously[13] and a predominant interaction with the rat liver RNAs was found. The results obtained in the in vitro experiments previously[14] and in this paper are in good correlation with the in vivo studies, as in all the cases the preferencial binding of Sudan I metabolites to the RNA was found. Because of the important role of RNAs in the translation of the genetic message[15], an evidence has been presented that changes in the structure of these nucleic acids (caused also by the binding of carcinogens) are associated with alterations in the metabolites regulation, cellular differentiation and neoplastic transformation, and of cell growth[15,16]. Thus, the effective modification of RNAs by activated Sudan I (besides the DNA) could also be implicated into the neoplastic processes due to this carcinogen. It was described earlier[14] that modification of tRNA by activated Sudan I increases the acceptor activity of tRNA for L-methionine. This modification may be important in the modulation of further processes of protein synthesis (increased protein formation) or in changing the regulatory processes (leading e.g. to the synthesis of aberrant proteins with modified biological functions)[14]. However, further extensive studies must be carried out to resolve these questions.

Microsomal proteins are the major targets for the binding of activated Sudan I, as the binding to these macromolecules is higher than to RNA or DNA when these nucleic acids are present in the reaction mixture. These proteins are, however, the most readily available macromolecules for modification in the cell, because of their locations. Hence, microsomal proteins (similarly as other proteins) which are present in the membrane of endoplasmic reticulum (cytochrome P-450) or in the cytoplasm (other proteins) can protect nucleic acids against the modification. The study of changes in the physiological functions of microsomal enzymes resulting from their modification with metabolites of Sudan I will be performed in order to gain results which could enlight the problems (i) how the modification of microsomal proteins can alter the cellular processes and (ii) whether it could be implicated in chemical carcinogenesis.

*We wish to express our gratitude to the late Professor D. Schmähl and to Dr E. Frei and Professor M. Wiessler from the Institute of Toxicology and Chemotherapy of the German Cancer Research Centrum in Heidelberg (F.R.G.) for the possibility to perform some experiments its laboratories and for the helpful scientific discussion.*

REFERENCES

1. *IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Man*, Vol. 8, p. 125. International Agency for Research on Cancer, WHO Monograph Sci., Lyon 1974.
2. Garner R. C., Martin C. N., Clayson D. B. in: *Chemical Carcinogens* (CH. E. Searle, Ed.), 2nd ed., Vol. 1, p. 175. American Chemical Society, Washington D. C. 1984.
3. Stiborová M., Asfaw B., Anzenbacher P., Lešetický L., Hodek P.: Cancer Lett. *40*, 319 (1988).
4. Stiborová M., Asfaw B., Anzenbacher P., Hodek P.: Cancer Lett. *40*, 327 (1988).
5. Rogg H., Wehrli W., Staehelin M.: Biochim. Biophys. Acta *195*, 13 (1969).

6. Matrka M., Pípalová J.: Collect. Czech. Chem. Commun. *47*, 2711 (1982).
7. Kimura T., Kodama M., Nagata Ch.: Gann *73*, 455 (1982).
8. Stiborová M., Asfaw B., Anzenbacher P.: Collect. Czech. Chem. Commun. *54*, 2021 (1989).
9. Kadlubar F. F., Miller J. A., Miller E. C.: Cancer Res. *38*, 3628 (1978).
10. Bradford M. M.: Anal. Biochem. *72*, 248 (1976).
11. Stiborová M., Asfaw B., Anzenbacher P.: FEBS Lett. *232*, 387 (1988).
12. Weinstein I. B.: Bull. N.Y. Acad. Med. *54*, 366 (1978).
13. Asfaw B., Stiborová M., Anzenbacher P.: *Int. Symp. Biochemistry of Chemical Carcinogenesis, Prague 1988*; Abstr. p. 1.
14. Stiborová M., Frei E., Klokow K., Wiessler M., Šafařík L., Anzenbacher P., Hradec J.: Carcinogenesis *11*, 1789 (1990).
15. Weistein L. B., Friedman S. M., Ochoa M., jr. : Cold Spring Harb. Symp. Quant. Biol. *31*, 671 (1967).
16. Weistein I. B.: Cancer Res. *28*, 1871 (1968).

Translation revised by H. P. Mašková.