Alkylation of Cellular Macromolecules by Reactive Metabolic Intermediate of DBCP*

Yasuhiro KATO, Kiyoshi SATO, Osami MATANO and Shinko GOTO

Institute of Environmental Toxicology, Kodaira, Tokyo 137, Japan

(Received June 7, 1979)

Alkylating potential of DBCP on cellular macromolecules *in vitro* and *in vivo* was investigated using ^{14}C -DBCP and male Wistar rats. In studies *in vitro* with rat-liver-microsome system, incorporation of DBCP-radiocarbon into TCA- and organic-solvents unextractable microsomal proteins was determined. The incorporation was dependent on incubation time, ^{14}C -DBCP concentration, enzymatically active microsomes, NADPH, and oxygen, but it was not affected by sufficient puromycin to inhibit protein synthesis. Addition of SKF-525A, diethylthiocarbamic acid, cysteine, or glutathione prevented the incorporation, and low concentration of 1,1,1-trichloropropane-2,3-oxide, an inhibitor of epoxide hydase stimulated the incorporation. When ^{14}C -DBCP (20 mg/kg) was orally administered to rats, TCA- and organic-solvents unextractable macromolecular residues were formed in rat tissues in parallel to the depletion of hepatic glutathione. Gel-filtration, protease digestion, and 2,4-dinitrofluorobenzene treating of the liver homogenate showed an existence of radioactive proteins of which primary structure was radiolabeled. DNA isolated from the liver was also radiolabeled. From these results it was concluded that DBCP is oxidatively metabolized to reactive metabolic intermediate(s) by microsomal cytochrome p-450 system and covalently bind with nucleophilic sites of the cellular macromolecules *in vitro* and also *in vivo*. And epoxide was proposed as one of the reactive metabolic intermediate(s) responsible for protein alkylation by DBCP.

INTRODUCTION

Incidence of sterility in male workers of DBCP-manufacturing plants¹⁾ attracted much interest of toxicological researchers. Experimental adverse effects of the chemical in various tissues including the sperm cells have been disclosed in several different species of animals.²⁻⁴⁾ Carcinogenicity studies revealed that stomach cancer is produced in rats and mice.^{5,6)} Mutagenicity has also been proven in bacterial systems,⁷⁾ and recently dominant lethality in rats was added as one of its genetical adverse effects.^{8,9)}

In a preceding paper on metabolic fate of DBCP in male rats we originally reported that after dosing rats with ^{14}C -DBCP, there were radioactive residues in target organs including the testes which were not extractable with

organic solvents and were suggested as alkylated cellular macromolecules.¹⁰⁾

During the past decade it has become increasingly evident that most of the chemicals having a potential to bind covalently with proteins also bind with nucleic acids¹¹⁾ and may cause cellular necrosis,¹²⁾ carcinogenicity,¹³⁾ and mutagenicity¹⁴⁾ (chromosomal aberration¹⁵⁾ and dominant lethality^{15,16)}, covering all of the adverse effects found with DBCP.

It is of our great concern to understand the biochemical transformation of DBCP leading to a genetical implication in mammals. And the investigation of its alkylating potential may be an important aspect to clarify the molecular mechanism of the genetic-damage induction.

Recently we evidenced the alkylation of proteins in rat liver-9000 g supernatant fortified with ^{14}C -DBCP.¹⁷⁾ In this paper radioactive incorporation into proteins and nucleic

* Metabolic Fate of DBCP in Rats (Part 2)

acids *in vivo* and some informations on the mechanism of protein alkylation *in vitro* were presented.

MATERIALS AND METHODS

1. Chemicals

1,2-Dibromo-3-chloropropane-3- ^{14}C (^{14}C -DBCP) of 94% radiochemical purity was prepared in New England Nuclear Co. in Boston. Pure non-radioactive 1,2-dibromo-3-chloropropane (DBCP) was prepared in this laboratory as cited in our preceding paper.¹⁰⁾ Pronase (type VI, from *Streptomyces griseus*), ribonuclease-B (RNase, type XII-B, from bovine pancreas), puromycin dihydrochloride, isocitrate dehydrogenase, and diethyldithiocarbamic acid (DEDTC) were purchased from the Sigma Chemical Co. Neutral protease (from *B. subtilis*), 1,2-epoxy-3,3,3-trichloropropane (trichloropropene oxide), and bis-(3-carboxy-4-nitrophenyl)-disulfide (DTNTB) were the products of Kanto Chemicals Co., Aldrich Chemicals Co., and Wako Pure Chemical Co., respectively. SKF-525A was kindly offered from Smith and Kline French Laboratories in Philadelphia.

2. Animals

Male Wistar Imamichi rats of 9 weeks of age were used throughout the experiments. In the experiments *in vivo* they were orally treated with 24.5 or 70.5 μCi (for DNA isolation)/20 mg/kg of ^{14}C -DBCP in olive oil and housed as described previously.¹⁰⁾

3. Analytical Procedures

3.1 Binding experiment *in vitro*

Reaction vessels (10 ml) contained the followings in a final volume of 1 ml of 0.1 M Tris-HCl (pH 7.4): male rat-liver-microsomal proteins (3.0 mg of microsomal proteins, determined by Biuret method) with or without 5-min boiling, NADPH-generating system (10 μmol of NADP^+ , 1 IU of isocitrate dehydrogenase, 8 μmol of isocitric acid disodium), 5 μmol of magnesium chloride, 0.241 μmol of ^{14}C -DBCP in ethanol (20 μl , 1.44×10^6 dpm), and 0.01 to 10 μmol of test substances (puromycin, SKF-525A, DEDTC, cysteine, glutathione, or trichloropropene oxide). Reaction was started by the addition of ^{14}C -DBCP, carried out

aerobically at 37°C for 60 min in a shaking water bath, and stopped by 9 ml of 5% trichloroacetic acid (TCA). In some instances the atmosphere in the reaction vessels was exchanged by nitrogen gas, and the incubation was carried out with sealing caps.

3.2 Isolation of hemoglobin

Rat hemoglobin was precipitated from the hemolysate of the blood cells collected 24 hr after dosing in according to Drabkin.¹⁸⁾

3.3 Determination of "bound" radiocarbon

Tissue homogenates (in 3 volumes of methanol), hemoglobin precipitated and *in vitro* incubation mixtures were exhaustively extracted (practically 2 to 5 times with more than 10 volumes of the following each solvent) with 5% TCA, 70% ethanol, acetone-chloroform mixture (4 : 1, v/v), and 80% methanol. Completion of the extraction was determined by monitoring the released radioactivity at each of the extraction steps. The residues obtained from the tissue homogenate and hemoglobin pellet were suspended in 70% methanol, and aliquots of the suspension were air-dried overnight on cellulose powder in combustor (Packard) followed by combustion.

Those prepared from the samples *in vitro* were digested with 2 ml of NCS tissue solubilizer (Amersham & Searl) for liquid scintillation counting.

3.4 Preparation of crude protein from the liver and kidney

The 24-hr liver and kidney were collected after a cardiac perfusion with 0.05 M sucrose in physiological saline. Their homogenates in 5 volumes of 0.05 M phosphate buffer, pH 7.0, were centrifuged at 3,000 g for 10 min, and the supernatants (5 ml) were gel-filtrated through Sephadex G-10 or G-50 columns (2.6 cm i.d., 45 cm gel length; elution buffer: 0.05 M phosphate buffer, pH 7.0; flow rate: 0.5 ml/min). Radioactive eluant in the blue dextran fraction (60-90 ml) from Sephadex G-10 column was repeatedly extracted (3 times with each solvents) with 5% TCA, 80% methanol, and acetone, successively. Crude protein residues thus obtained were refluxed with more than 50 volumes of methanol, acetone, and chloroform, successively. Finally prepared crude protein residues were processed to an enzymatic hydrolysis.

3.5 Detection of radioactive amino acids in hepatic proteins

Hepatic crude protein (500–900 mg, dry weight) prepared as above was digested for 24 hr by 5,000 PUNs of protease at 37°C in 0.1 M phosphate buffer, pH 7.5. Half of the hydrolysate was exhaustively extracted with half volumes of ethyl ether under basic (pH 8.4) and acidic (pH 1.0) conditions, in this order. Remaining half was lyophilized and treated with 5% 2,4-dinitrofluorobenzene (DNFB, 25 ml) for 4 hr in 5% sodium bicarbonate solution. Then the reaction mixture was extracted with ethyl ether as indicated above.

3.6 Isolation of hepatic DNA

Hepatic DNA was isolated from 3 rats withdrawn 24 hr after dosing by the method described by Tano *et al.*¹⁹⁾ Briefly, the process involved protein removal by phenol shake after treatment with 6% *p*-aminosalicylic acid sodium. After precipitation of the partially purified DNA with ethoxyethanol, the samples were redissolved in $0.1 \times \text{SSC}$ (1.5 M sodium-chloride + 0.15 M citrate trisodium) and incubated with pronase (1,000 PUNs, self-digested for 40 min at room temperature before use). Polysaccharides were removed by shaking with 1.25 M phosphoric acid and 2-methoxyethanol. Then DNA samples were dialyzed overnight against distilled water and treated with ethoxyethanol. Precipitated DNA was again dissolved in 0.01 M Tris-HCl (pH 7.3) and incubated with 160 Us of RNase (80°C, for 10 min before use) and 100 PUNs of pronase, in this order. The sample was again purified by an ethoxyethanol-precipitation and by an overnight-dialysis against distilled water. Then the bulk volume of the DNA sample was reduced by Lyphogel (German Inst. Co., Michigan) and collogion bags. Aliquots of the purified DNA solution were poured onto cellulose powder for combustion, and optical density scans were run to verify the purity of the DNA by Hitachi spectrophotometer model 139.

3.7 Glutathione determination

The livers were excised and homogenized in 4.5 volumes of ice-cold 0.1 M phosphate buffer, pH 7.4. Glutathione was determined by DTNTB method.²⁰⁾ The protein in an aliquot of the liver homogenate was precipi-

tated by an equal volume of 4% sulfosalicylic acid. And 0.2 ml of the supernatant was added to 4.8 ml of DTNTB reagent (0.1 mM DTNTB in 0.1 M phosphate buffer, pH 8.0). Reduced glutathione was proportional to the absorbancy at 412 nm.

3.8 Radioassay

Combustion prior to liquid scintillation counting was performed by Packard TriCarb 306 sample oxidizer and Aloka ACS 113 auto-combustion system.²¹⁾ Radioactivity was determined by Aloka 903 liquid scintillation counter using PCS (Amersham and Searle) as scintillation liquid. Quenching level was estimated by an external standardization method in most instances, and in some cases with respect to the samples containing DNFB or its derivatives it was determined by an internal standard method.

RESULTS

1. Alkylation of Microsomal Proteins *in vitro*

Radiocarbon due to ^{14}C -DBCP was incorporated into the rat-liver-microsomal proteins *in vitro*. Effects of the incubation time and substrate concentration are shown in Fig. 1. This incorporation required the native microsome and NADPH. Oxygen seems also an essential factor, and the incorporation was partially prevented under the incomplete anaerobic atmosphere. Requirement for an oxidative metabolic transformation of DBCP on microsome was further evidenced by SKF 525A as shown in Table 1. Puromycin, an inhibitor for protein synthesis (K_m for rat liver peptidyl transferase: $8 \mu\text{M}$ ²²⁾) did not show any significant preventing effect on the incorporation, even with its mM orders. This result indicates that the incorporation is not due to the metabolic formation of endogenous substrates for macromolecules, but to an irreversible covalent binding of a reactive metabolic intermediate with microsomal proteins (alkylation of microsomal proteins).

Cysteine and glutathione prevented the covalent binding. The low concentration of trichloropropene oxide, an inhibitor of microsomal epoxide hydrolase possessing a highly electrophilic property,²³⁾ increased the alkylation by 1.24 folds. However, as its concentration increased, it became to show a prevent-

ing effect. A free radical scavenger,²⁴⁾ DEDTC acted as an inhibitor.

Lineweaver-Burk plots obtained from the double-reciprocal analysis of the data in Fig. 1-B deduced the following minimum-square-regression line: $[1/V_{\text{nmol DBCP/60 min /mg microsomal protein}}] = 0.145 \times [1/S_{\text{mM DBCP}}] + 0.4698$ ($\gamma = 0.9996$). Therefore, the K_m and V_{max} were estimated as 2.8 mM and 2.1 nmol DBCP bound/60 min/mg microsomal protein.

2. Formation of Radioactive "Alkylated Macromolecular Residues" and Depletion of Hepatic Glutathione *in vivo*

Formation of the TCA- and organic-solvents unextractable residues reached maximum as early as 6 hr after dosing (Table 2). The occupation of the radioactivity by that residues in tissue whole radioactivity increased with the elapse of time. In parallel to the radioactive residue formation, a depletion of hepatic glutathione was observed at early stages after dosing as shown in Table 3.

3. Characterization of the "Alkylated Residues"

Blood-removed homogenates (3,000 g supernatant) of the 24-hr liver and kidney were

gel-filtrated through Sephadex G-10 columns. Approximately 80% of the radioactivity in the homogenates were eluted in the blue dextran fraction, more than half of which radioactivity were recovered as TCA- and organic-solvents unextractable radioactive residues. This residue sample was again gel-filtrated through the Sephadex G-10 column after the digestion by protease to give no radioactive recovery in the blue dextran fraction (Table 4).

Similar to the radioactive hepatic protein formation, approximately 40% of the radiocarbon in the blood cell associated with hemoglobin (Table 5). In these experiments removal of the noncovalently bound radiocarbon from the protein residues was considered practically complete, and no further release of radiocarbon was found by the successive reflux with dichloromethane, acetone, and methanol.

Further evidence for the covalent nature of the interaction between the radioactive moiety of ^{14}C -DBCP and proteins was achieved by a derivatization of the protease-hydrolysate of hepatic proteins to 2,4-dinitrophenyl amino acids by DNFB. While radiocarbon in the hydrolysate was not extracted into ethyl

Table 1 Irreversible covalent binding of ^{14}C -DBCP with microsomal proteins *in vitro*.

Incubation mixture		Bound radiocarbon (nmol bound/60 min/3 mg protein)	
Basic system		0.498±0.053	100%
— native microsome (boiled)		0.012±0.009**	2%
— NADPH generating system		0.016±0.010**	3%
— oxygen (under nitrogen)		0.275±0.031**	55%
+ SKF 525A	0.1 mM	0.168±0.024**	34%
	1.0 mM	0.028±0.011**	6%
+ puromycin	0.1 mM	0.514±0.058	
	1.0 mM	0.453±0.034	
+ glutathione	1.0 mM	0.126±0.016**	25%
+ cysteine	1.0 mM	0.188±0.021**	38%
+ trichloropropene oxide			
	0.01 mM	0.617±0.042**	124%
	0.1 mM	0.482±0.063	
	1.0 mM	0.360±0.027**	72%
+ DEDTC	1.0 mM	0.151±0.055**	30%

The basic system (1 ml) contained rat liver microsomes (3 mg protein/ml); Tris-HCl buffer, pH 7.4 (100 mM); ^{14}C -DBCP (0.24 μmol); NADPH-generating system. Reaction was started by the addition of substrate in 20 μl ethanol and incubated under air for 60 min with shaking at 37°C.

Values are mean $\pm$ S.D. ($n=6-12$) ** : $p<0.01$

Table 2 Formation of radioactive bound residue in male rat tissues after a single oral dosing of ^{14}C -DBCP (20 mg/kg).

Rat tissue	Time after dosing		
	6 hr	24 hr	48 hr
	ppm equivalent of ^{14}C -DBCP		
Blood cells	3.01 ± 0.09^a	1.54 ± 0.18^a	1.39 ± 0.21^a
	1.73 ± 0.22^b (57.5%)	1.03 ± 0.17^b (66.9%)	0.98 ± 0.19^b (70.5%)
Liver	20.92 ± 3.10^a	15.64 ± 3.81^a	7.95 ± 2.14^a
	14.71 ± 1.84^b (70.3%)	10.42 ± 2.90^b (66.5%)	6.21 ± 0.85^b (78.2%)
Kidney	30.13 ± 4.31^a	9.08 ± 1.64^a	7.20 ± 1.04^a
	12.51 ± 2.19^b (41.5%)	6.02 ± 1.70^b (66.3%)	4.99 ± 0.92^b (69.3%)

^a: whole radiocarbon level in tissue.^b: TCA- and organic-solvents unextractable bound residue.(): $100 \times [b/a]$ Values are mean $\pm$ S.D. of 4 rats.Table 3 Depletion of hepatic glutathione level after a single oral dosing of ^{14}C -DBCP (20 mg/kg).

Duration (hr)	Glutathione level (mm in fresh liver)	
0	8.02 ± 0.13	(100%)
6	$6.14 \pm 0.55^{**}$	(77%)
24	7.74 ± 0.32	

Glutathione was determined by DTNTB method. Values are means $\pm$ S.D. of 6-8 rats. **: $p < 0.05$

Table 4 TCA-insoluble radioactive macromolecular residues in the liver and kidney.

Fraction	Radioactivity recovered (% of 3,000 g supernatant)	
	Liver	Kidney
3,000 g supernatant	100	100
Sephadex G-10 BDF*(A)	79.8	82.0
Sephadex G-50 BDF*	62.1	60.3
TCA- and organic solvents-insoluble residue of A(B)	59.9	50.8
Sephadex G-10 BDF* of protease hydrolysate prepared from B**	<2	<2

Blood removed tissues collected 24 hr after dosing (20 mg/kg of ^{14}C -DBCP) were homogenized and centrifuged at 3,000 g for 10 min.

BDF*: blue dextran fraction through Sephadex G-10 or G-50 columns.

**: TCA- and organic-solvents insoluble residue prepared from Sephadex G-10 DBF was digested by protease and again gel-filtrated through Sephadex G-10 column.

Values are mean of 3 experiments.

Table 5 Radioactive incorporation of ^{14}C -DBCP into rat hemoglobin.

Fraction	% of radioactivity in the blood cells
Toluene layer	0.61 ± 0.18
Cell sap.	26.94 ± 4.37
Stroma	6.82 ± 4.00
Hemoglobin pellet	59.32 ± 3.08
TCA- and organic solvents treated hemoglobin*	40.11 ± 2.41

The blood cells (24 hr after oral dosing of ^{14}C -DBCP, 20 mg/kg) was fractionated according to D. L. Drabkin (1949).

*: Hemoglobin pellet was washed with 5% TCA and exhaustively extracted with 80% methanol and acetone-chloroform (4 : 1).

Values are mean $\pm$ S.D. of 4 rats.

ether from either acidic or basic solution, 68% of the radioactivity could be extracted into ethyl ether under acidic but not basic condition by the DNFB treating, indicating the formation of proteins of which primary structure was radiolabeled (Table 6).

4. Radioactive Incorporation into Hepatic Macromolecular Nucleic Acids

DNA samples prepared from the 24-hr liver was considered free from ^{14}C -DBCP and its unbound metabolites through the repeated wash with dilute salt solution, phenol, and ethoxyethanol involved in its isolation proc-

esses. Finally obtained protein-, RNA-, and polysaccharides-free hepatic DNA (Fig. 2, Table 7) included low but significant radioactivity (0.03% of liver radiocarbon). When RNase- and the 2nd pronase treating were omitted from the isolation processes, presumably giving RNA-contaminated DNA or chromatin, 1.4% of the hepatic radioactivity was recovered as "DNA"-associated radiocarbon.

DISCUSSION

Enzymatic alkylation of proteins with DBCP has previously been reported by using rat-liver 9,000 g supernatant.¹⁷⁾ In this paper we have reconfirmed the alkylation of microsomal proteins by DBCP *in vitro* using sufficient puromycin to inhibit protein synthesis.²²⁾

Fig. 1 Covalent binding of ^{14}C -DBCP with microsomal proteins.

A: time course (0.241 mM of ^{14}C -DBCP)

B: effect of substrate concentration (incubation time: 60 min)

Liver microsome (3 mg of protein) from male rats was incubated with ^{14}C -DBCP in the presence of an NADPH-generating system under air at 37°C.

Each point represents mean $\pm$ S.D. of 5 to 6 experiments.

Maximum alkylation velocity with DBCP was approximately 4.8 folds of that observed with vinylchloride (0.44 nmol bound/hr/mg microsomal protein).²⁵⁾ The alkylation primarily required an oxidative metabolic activation by

Fig. 2 UV spectra of isolated hepatic DNA in $0.01 \times \text{SSC}$.

Table 6 Extraction of radioactive components from protease-digested hepatic proteins.

Treatment with DNFB*	Radioactive recovery in ethylether under	
	Basic (pH 8.4)	Acidic (pH 1.0)
—	0.2%	7.5%
+	5%	68.4%

*: 2,4-Dinitrofluorobenzene (DNFB) was reacted with protease-digested proteins which had been exhaustively washed and extracted with 5% TCA and organic solvents. Values are means of duplicate (DNFB-) or triplicate (DNFB+) experiments.

Table 7 Incorporation of DBCP radiocarbon into hepatic DNA after an single oral dosing of ^{14}C -DBCP (20 mg/kg).

DNA sample	% of liver radioactivity	[OD _{260nm} : OD _{230nm} : OD _{280nm}]
Purified DNA*	0.029 (n=2)	1.000 : 0.502 : 0.498
RNA- and protein-contaminated DNA**	1.36 (n=3)	1.000 : 0.672 : 0.554

*: DNA was isolated from the liver (24 hr after dosing) according to the method described by Tano, *et al.* (1979).

**: Not treated by RNase and pronase.

microsomal cytochrome P-450 system. Inhibition of the alkylation *in vitro* by sulfhydryl containing compounds is explained by a known function of these compounds that being alkylated themselves they protect vital nucleophilic sites in tissues from an electrophilic attack by alkylating intermediates of chemicals²⁶⁾ and as their concentrations are depleted, the alkylating intermediates are more likely to react with cellular macromolecules, eliciting proportionally greater toxicity.²⁷⁾ This concept is in agreement with our previous observation that DBCP is readily excreted in the bile and urine as a form of an amphoteric-natured and ninhydrin-positive conjugate which was suggested as a glutathione conjugate. From these observations it may be suggested that the reactive metabolic intermediate of DBCP possesses an affinity for sulfhydryl and other nucleophilic residue groups in proteins.

In regard to the reactive metabolic intermediate of DBCP, an involvement of some epoxide is proposed from the finding that 10 μM of trichloropropene oxide, an inhibitor of epoxide hydrase,²⁸⁾ promoted the alkylation *in vitro* as reported with vinyl chloride.²⁵⁾ This proposal seems in agreement with the above mentioned inhibitory effect of glutathione, because epoxides are known enzymatically and nonenzymatically to bind with glutathione.²⁸⁾ Preventing effect of the high concentrations of trichloropropene oxide on the protein alkylation by DBCP could be explained as that at high concentrations it acts as a competitive inhibitor for the protein alkylation by DBCP rather than acts as a promoter by inhibiting epoxide hydrase, since the chemical is highly electrophilic and it is also able to bind with nucleophilic sites of microsomal proteins. If V_{max} obtained is postulated to represent all of the number of nucleophilic sites in microsomal proteins, the reactive intermediate formed from 240 nmol of DBCP would compete with 10 to 1,000 nmol of trichloropropene oxide for their common bindings sites (6.4 nmol/3 mg microsomal protein) in our experimental conditions.

Inhibitive effect of DEDTC shows an involvement of a free radical in the processes of the reactive intermediate formation and subsequent protein binding. However, it is

unconceivable which step DEDTC inhibits, because a free radical scavenger, DEDTC is reported to inhibit the metabolism of some foreign compounds on microsome.^{24,29,30)} And a conclusion on this free-radical problem was brought to further works.

Recently it has become increasingly evident that relative part of haloalkanes and haloalkenes possesses a potential to alkylate proteins, RNA, and DNA *in vitro* and also *in vivo* (eg., ethylenedibromide,³¹⁾ methylenechloride,³²⁾ vinylchloride,^{25,33)} vinylbromide³⁴⁾). Alike these chemicals radiocarbon due to ^{14}C -DBCP was incorporated into the TCA- and organic solvents unextractable macromolecules majorly consisted of protein, DNA, and presumably RNA, in association with the depletion of hepatic glutathione *in vivo*.

These results *in vivo* and *in vitro* permit the following conclusion that DBCP is converted to a reactive metabolic intermediate by an oxidative metabolic activation on microsome and alkylates the cellular macromolecules *in vitro* and *in vivo*.

This conclusion is consistent with the results from the mutation study that DBCP induces a base-substitution type of mutation in *Salmonella*.⁷⁾

Among the experimental adverse effects of DBCP, a correlation between necrotic incidence⁴⁾ and protein alkylation or covalent binding of xenobiotics with proteins has been well elucidated in this decade.^{12,27)} In regard to the dominant lethality^{8,9)} and chromosomal aberration,³⁵⁾ G. A. Sega and J. G. Owens has recently deduced an interesting conclusion from their work on molecular dosimetry of ethyl methanesulfonate (a well known alkylating agent, EMS) in mice germ cells.¹⁶⁾ They proposed a model that ethylation of cysteine sulfhydryl groups contained in mouse-sperm protamine could block normal disulfide-bond formation, preventing proper chromatin condensation in the sperm nucleus. Stresses in the chromatin structure could then eventually lead to chromosome breakage, with resultant dominant lethality. These citations and the results presented herein and previously make us suspect a close correlation between the alkylating potential of DBCP and its toxicological implications, especially the genetic

damage.

REFERENCES

- 1) *Chem. Eng. News*, Aug. 22 (1977)
- 2) T. R. Yorkelson, S. E. Sadek, V. K. Rowe, J. K. Kodama, H. H. Anderson, G. S. Loquram & C. H. Hine: *Toxic. Appl. Pharmac.* **3**, 545 (1961)
- 3) E. V. Faidysh, N. N. Rahnatullaev & V. A. Varshvskii: *Med. Zh. Uzb.* **1**, 64 (1970)
- 4) Ya. B. Reznic & G. K. Spichan: *Gig. Sanit.* **6**, 101 (1975)
- 5) W. A. Olson, R. T. Habermann, E. K. Weisburger, J. M. Ward & J. H. Weisburger: *J. Natl. Cancer Inst.* **51**, 1993 (1973)
- 6) M. B. Powers, R. W. Voelker, N. P. Page & K. Weisburger: *Toxic. Appl. Pharmac.* **33**, 171 (1975)
- 7) H. C. Rosenkranz: *Bull. Environ. Contam. Toxicol.* **14**, 8 (1975)
- 8) K. S. Rao, F. J. Murray, A. A. Crawford, J. A. John, W. J. Potts, B. A. Schwetz, J. D. Burek & C. M. Parker: Abstract of 18th Annual Meeting of the Society of Toxicology, A-137 (No. 273), March, 1979
- 9) S. Teramoto, R. Saito, H. Aoyama & Y. Shirasu: *Mutation Res.* **77**, 71 (1980)
- 10) Y. Kato, K. Sato, S. Maki, O. Matano & S. Goto: *J. Pesticide Sci.* **4**, 901 (1979)
- 11) J. R. Gillette: *Biochem. Pharmac.* **23**, 2785 (1974)
- 12) J. R. Gillette & L. R. Pohl: *J. Toxicol. Environ. Health* **2**, 849 (1977)
- 13) J. A. Miller: *Cancer Res.* **30**, 559 (1970)
- 14) A. Léonard: in "Chemical Mutagens: Principles and Methods for their Detection" ed. by A. Hollaender, Vol. III, p. 21, Plenum, New York, 1971
- 15) U. H. Ehling, R. B. Cumming & H. U. Malling: *Mutation Res.* **5**, 417 (1968)
- 16) G. A. Sega & J. G. Owens: *Mutation Res.* **52**, 87 (1978)
- 17) Y. Kato, O. Matano & S. Goto: *Toxic. Lett.* **3**, 299 (1979)
- 18) D. L. Drabkin: *Arch. Biochem.* **21**, 224 (1949)
- 19) S. Tano, S. Mizuno & S. Shirahata: *Biochem. Biophys. Acta* **213**, 45 (1970)
- 20) G. L. Ellman: *Arch. Biochem. Biophys.* **82**, 70 (1959)
- 21) Y. Kato, K. Sato, O. Matano & S. Goto: *J. Pesticide Sci.* **3**, 27 (1978)
- 22) S. Pestka, G. Rakesh, H. Rosenfeld, N. Claire & H. Henry: *J. Biol. Chem.* **247**, 4258 (1972)
- 23) F. Oesch: *Xenobiotica* **3**, 305 (1972)
- 24) L. M. Lutz, E. A. Glende, Jr. & R. O. Recknagel: *Biochem. Pharmac.* **22**, 1729 (1973)
- 25) H. Kappus, H. M. Bolt, A. Buchter & W. Bolt: *Toxic Appl. Pharmac.* **37**, 461 (1976)
- 26) J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette & B. B. Brodie: *J. Pharmacol. Exp. Therap.* **187**, 211 (1973)
- 27) J. R. Gillette, J. R. Mitchell & B. B. Brodie: *Ann. Rev. Pharmacol.* **14**, 271 (1974)
- 28) E. Boyland: "Concepts of Biochemical Pharmacology," ed. by B. B. Brodie and J. R. Gillette, vol. 23, p. 548, Springer-Verlag, Berlin, 1971
- 29) T. Sakaguchi, N. Hishimura, K. Masuda, I. Tuge, K. Onishi & T. Tusami: *Biochem. Pharmac.* **15**, 756 (1966)
- 30) S. E. Abanobi, J. A. Popp, S. K. Chang, G. W. Harrington, P. D. Lotlikar, D. Hadjiolov, M. Levitt, S. Rajalakshmi & D. S. R. Sarma: *J. Natl. Canc. Inst.* **58**, 263 (1977)
- 31) E. Nachtomi & D. S. R. Sarma: *Biochem. Pharmac.* **26**, 1941 (1977)
- 32) G. D. DiVincenzo & M. L. Hamilton: *Toxic. Appl. Pharmac.* **32**, 385 (1975)
- 33) P. G. Watanabe, J. A. Zempel, D. G. Pegg & P. J. Gebring: *Toxic. Appl. Pharmac.* **44**, 571 (1978)
- 34) H. M. Bolt, J. G. Filser & R. K. Hinderer: *Toxic. Appl. Pharmac.* **44**, 481 (1978)
- 35) M. Tezuka: submitted for *Mutation Res.*

要 約

DBCP 活性代謝中間体による生体高分子物質のアルキル化

加藤保博, 佐藤 清, 俣野修身, 後藤真康

DBCP による生体高分子物質のアルキル化を ^{14}C -DBCP とウィスター系雄ラットを用い調査した。ラット肝 microsome を用いた *in vitro* の実験では TCA および有機溶媒で抽出されない microsome 蛋白中への ^{14}C の取り込みを調べた。この取り込みは反応時間、基質濃度、活性 microsome, NADPH, 酸素に依存しており、また蛋白質合成阻害剤である puromycin により影響されない。SKF-525A, diethyldithiocarbamic acid, システイン, グルタチオンは阻害効果を示し epoxide hydase の阻害剤である 1,1,1-trichloropropane-2,3-oxide によって促進された。一方 20 mg/kg の ^{14}C -DBCP をラットに経口投与した *in vivo* の実験では TCA および有機溶媒により抽出困難な放射性高分子残渣の形成が、肝グルタチオンレベルの低下と平行して認められた。この放射性残渣の実体は肝ホモジネートのゲル濾過、プロテアーゼ処理、2,4-dinitrofluorobenzene によ

る誘導体化によりその一次構造が放射化された蛋白を主体とするものであることが明らかとなった。また肝 DNA にも ^{14}C が取り込まれていた。以上の結果から本報では DBCP は肝 microsome の cyt. P-450 系により

酸化代謝を受けて活性代謝中間体を生じ、これが生体高分子物質の親核性残基と共有結合し、*in vitro*, *in vivo* でこれらをアルキル化するものと結論された。またこの活性中間体の一つとしてエポキシイドの関与を認めた。