Biochemical Pharmacology, Vol. 20, pp. 135–139. Pergamon Press, 1971. Printed in Great Britain

MECHANISM OF "BIOCHEMICAL SHOCK"—II

DIRECT DEMONSTRATION OF MIXED DISULFIDE INVOLVEMENT IN THE INHIBITION OF DEOXYCYTIDINE FORMATION IN RAT THYMUS *IN VIVO*

I. V. FILIPPOVICH, T. N. SHEREMETYEVSKAYA and E. F. ROMANTZEV
Institute of Biophysics USSR Ministry of Health, Moscow, USSR

(*Received* 23 *April* 1970; *accepted* 24 *June* 1970)

Abstract—The addition of β-mercaptoethylamine (MEA), cystamine,2-mercaptoethanol, D-penicillamine and *p*-chloromercuribenzoate to the partially purified deoxyribose-transferring enzyme (EC 2.4.2.6) from rat thymus gland leads to acute inhibition of thymidine phosphorylase and deoxyribose transferase activities of the enzyme. This inhibition may depend on the reaction of these substances with protein SH- and SS-groups. Fifteen min after i.p. administration of ^{35}S-MEA, enzyme labelled with MEA was isolated. Both enzymatic activities were depressed (by 57–60 per cent). Treatment of this inhibited enzyme with DTT (Dithiothreitol) liberates labeled MEA and simultaneously leads to a partial recovery of both activities. The residual inhibition was 25–30 per cent of the control. It may be concluded that the inhibition in deoxycytidine formation in rat thymus after i.p. MEA administration at the time when its pronounced radioprotective effect is obvious can be explained at least partially by mixed disulfides formation between the radioprotector and the enzyme.

ONE OF the most promising hypotheses on the mechanism of action of radioprotective compounds is the hypothesis of "biochemical shock" advanced by Bacq *et al.*[1,2] At the same time little is known about the molecular mechanisms of "biochemical shock" i.e. about the effect of radioprotectors on various metabolic processes in mammals and, especially on the process of DNA biosynthesis.

As was shown in our earlier communications[3,4] the process of deoxycytidine formation from cytosine (as the acceptor of deoxyribose) and thymidine (the donor of deoxyribose) via phosphorylase-transferase reactions in rat thymus is sharply inhibited 15–20 min after i.p. MEA administration at a radioprotective dose. In our first communication[5] we demonstrated that the inhibitory action *in vivo* of some thiol radioprotectors on various biochemical processes correlates with their maximal accumulation in tissues and protective effectiveness. It was supposed that one of the possible reasons for such inhibition may be the mixed disulfides formation between the radioprotector and appropriate enzymes. Later we succeeded in showing that the temporal inhibition of deoxycytidine formation in thymus after MEA administration coincides in time with maximal accumulation of mixed disulfides of MEA with soluble proteins of thymus hyaloplasm.[6] Therefore it was supposed that sharp inhibition of deoxycytidine formation observed in rat thymus after MEA administration was conditioned, at any rate partly, by mixed disulfides formation of radioprotector with enzymatic systems responsible for this process.

The present study was undertaken to demonstrate that direct interaction of MEA with the appropriate enzyme *in vivo* may be one of the possible reasons for the inhibition observed, and so for the "biochemical shock" produced.

MATERIALS AND METHODS

Animals

Male albino rats (150–180 g) were used in the experiments and maintained on ordinary ration and water *ad lib.*

Reagents

MEA-bitartrate (>98 per cent) and ^{35}S-MEA (sp.act.67 mc/g, 98–99 per cent purity) were used. For the assay of transferase activity [2^{14}C]-cytosine (sp. act. 21 mc/g) and unlabeled thymidine ("Calbiochem") were employed. Dithiothreitol (DTT) was obtained as described earlier.[5] D-penicillamine hydrochloride was purchased from "Calbiochem", and *p*-chloromercuribenzoate from "Chemapol", Czechoslovakia.

Experimental procedures

Unlabeled MEA-bitartarate was administered i.p. at a dose of 150 mg/kg (as a free base). The rats were decapitated 15–20 min after the injection. The partial purification (up to 380-fold) of thymidine:cytosine deoxyribosyl transferase (EC 2.4.2.6.) from thymus gland was made according to the method[7] for human leukocytes and will be described elsewhere.[8] In the experiments with MEA-^{35}S each rat received 0·5 mc of isotope together with unlabeled radioprotector. In this case the animals were also sacrificed 15–20 min later and the purification of labeled enzyme was carried out as in the experiments with unlabeled MEA. The activity of the enzyme isolated from control rats and those injected with MEA was assayed both on thymidine phosphorolysis and on labeled deoxycytidine formation in the incubation systems containing [2^{14}C]-cytosine and unlabeled thymidine.[3,9]

The complete reaction mixture (0·4 ml) for the assay of thymidine phosphorylase activity contained: 16 μmoles of thymidine, 25 μmoles of NaH_2PO_4, 10 μmoles of Tris-HCl (pH 7·4) and 20 μg of partially purified enzyme fraction. After incubation at 37° for 60 min the reaction was terminated by quickly adding 1 ml of 3 per cent $HClO_4$. To 0·5 ml of clear solution 2 ml 0·3 N NaOH was added, and the absorbancy at 300 mμ due to free thymidine was read. The reference cuvet was identical except the thymidine was omitted. The absorbancy was linear with protein concentration. The activity of phosphorylase was calculated from ΔE_{300} using the standard equation.[9] Activity of the enzyme was expressed in units per mg of protein.

For the assay of transferase activity the incubation mixture (0·5ml) contained: 1 μmole of [2^{14}C]-cytosine (1·2.10^5 c.p. 10^2 sec), 1 μmole of thymidine, 12 μmole of Tris-HCl (pH 7·4) and 20 μg of partially purified enzyme fraction. After incubation for 60 min at 37° the reaction was terminated by cooling the tubes in ice, and 0·05 ml of solution was spotted on Whatman No. 1 paper together with unlabeled deoxycytidine as a sample. The solvent system of choice was iso-propanol–HCl–water (170:41:39) in which deoxycytidine and thymidine have R_f-values of 0·62 and 0·86 respectively; R_f of cytosine was 0·46. The spot corresponding to deoxycytidine was located in u.v. and its radioactivity counted in a T-25 BFL counter. The activity of

transferase was expressed in c.p. 10^2 sec in deoxycytidine formed per mg of protein. As well as for phosphorylase the transferase activity was linear with protein concentration.

The treatment of the enzyme with DTT was carried out at standard conditions (Tris-HCl, pH 7·4, 15 min).[6] After the treatment the excess of DTT and free MEA were removed by dialysis (against Tris-HCl, pH 7·4 for 1 hr). In control experiments the inactivation of enzyme during the dialysis procedure did not exceed 10 per cent from the initial level (both in the phosphorylase and transferase activities).

RESULTS AND DISCUSSION

The effect of various additives on phosphorylase and transferase activities of partially purified enzyme fraction is shown in Table 1.

These experiments were made with three different samples of enzyme, differing by the degree of purification. All of the compounds inhibit both activities.

TABLE 1. EFFECT OF VARIOUS COMPOUNDS *in vitro* ON PHOSPHORYLASE AND TRANSFERASE ACTIVITIES OF PARTIALLY PURIFIED ENZYME FRACTION

Compound added		Activity of phosphorylase			Activity of transferase		
		Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
MEA, μmoles	0·001	110	115	110	107	110	107
	0·025	102	110	110	103	101	97
	0·050	86	95	92	86	83	—
	0·100	71	87	88	68	75	—
Cystamine, μmoles	0·001	106	100	97	104	105	103
	0·025	102	89	84	77	102	93
	0·050	49	78	74	38	66	—
	0·100	6	59	51	20	57	—
2-mercapto-ethanol, μmoles	0·001	100	102	—	100	—	—
	0·025	97	97	—	101	—	—
	0·050	65	54	—	43	81	—
	0·100	21	50	—	14	15	—
D-penicil-lamine, μmoles	0·001	110	95	97	101	97	97
	0·025	78	88	83	85	81	—
	0·050	69	59	66	64	70	—
	0·100	78	51	36	46	35	—
p-chloro-mercuri-benzoate	$4 \cdot 10^{-4}$M	13	—	—	44	—	—
Dithio-threitol	$7 \cdot 10^{-4}$M	92	—	—	95	—	—

The incubation mixtures contained 10 μg of the protein. The results were expressed in per cent of the control.

As one may conclude from the inhibitory action of *p*-chloromercuribenzoate the enzyme contains SH- (and probably SS-groups) responsible for its catalytic action. Therefore the inhibitory action of cystamine on both enzymatic activities probably may be explained by mixed disulphide formation between cystamine and SH-groups of the enzyme. The inhibitory action of thiols (MEA, 2-mercaptoethanol, D-penicillamine) may be connected with their interaction with SS-groups of the enzyme which are necessary for the stabilization of its molecule in catalytically active form as it takes place in the case of pepsin interaction with 2-mercaptoethanol.[10]

On the other hand the possibility of spontaneous thiols oxidation during the incubation cannot be excluded. In this case the appropriate disulfides formed may also interact with SH-groups of the enzyme.

The slight stimulatory action of MEA added at the amounts of 0·001–0·025 μmoles probably may be attributed to the antioxidative effect of the thiol on SH-groups of the enzyme. In this case the amount of mixed disulfide formed may be too negligible to exert the inhibitory action.

In the next series of experiments MEA-^{35}S or unlabeled MEA was injected into rats, 15 min later the animals were decapitated and isolation of partially purified enzyme was carried out. The results of these experiments are represented in Table 2.

TABLE 2. MIXED DISULFIDE FORMATION BETWEEN MEA AND DEOXYRIBOSE-TRANSFERRING ENZYME IN RAT THYMUS 15 min AFTER i.p. LABELED OR "COLD" MEA ADMINISTRATION AT A RADIOPROTECTIVE DOSE

Experimental conditions	Specific activity of the enzyme		Radioactivity of protein, ^{35}S c.p. 10^2 sec per total volume of fraction
	Phosphorylase, units per mg of protein	Transferase, c.p. 10^2 sec in deoxycytidine per mg of protein	
Control (no MEA was injected)	24·5	1,373,333	
15 min after MEA administration	13·0 14·8 (57%)	905,280 951,849 (60%)	385 110
After DTT treatment	19·0 19·3 (30%)	1,267,300 1,298,253 (25%)	255 60

The percentage inhibition is indicated in parenthesis.

MEA administration to rats leads to pronounced inhibition in both activities of the purified enzyme. This inhibition is caused by MEA bound to the protein because the purified enzyme is radioactive. After treatment with DTT, the radioactivity of this inhibited enzyme loaded with MEA is decreased. This points to the involvement of mixed disulfides in the inhibition observed *in vivo*. Moreover, after DTT treatment both enzymatic activities are partially recovered.

Therefore the inhibition in deoxycytidine formation in rat thymus after i.p. MEA administration observed earlier[4] is obviously connected partly with mixed disulphide formation between the appropriate enzyme and radioprotector. This inhibition coincides with the optimum of radioprotective action of MEA. It is quite probable

that the reversible effect of aminothiol on the enzymatic activity may be explained by the dissociation of mixed disulphides under the effect of disulfide-reducing system of the organism.[6]

Acknowledgement—The authors wish to thank Miss V. P. Orlova for excellent technical assistance.

REFERENCES

1. Z. M. Bacq and P. Alexander, *Nature, Lond.* **203,** 162 (1964).
2. Z. M. Bacq and R. Goutier, *Brookhaven Sympos. Biol.* **20,** 241 (1968).
3. I. V. Filippovich and E. F. Romantzev, *Voprosy Med. Chimii* (russ.). **16,** 355 (1968).
4. T. N. Sheremetyevskaya, I. V. Filippovich and E. F. Romantzev, *Voprosy Med. Chimii* (russ.). **16,** 437 (1970).
5. I. V. Filippovich, N. N. Koshcheenko and E. F. Romantzev, *Biochem. Pharmac.* in press (1970).
6. I. V. Filippovich, Z. I. Zhulanova, Z. A. Trebenok, T. N. Sheremetyevskaya and E. F. Romantzev, *Dokl. Akad. Nauk SSSR* (russ.). 195, 222 (1970).
7. R. C. Gallo and T. R. Breitman, *J. biol. Chem.* **243,** 4936 (1968).
8. I. V. Filippovich, T. N. Sheremetyevskaya and E. F. Romantzev, *Biokhimia* (russ.). in press (1971).
9. M. Friedkin and De Wayne Roberts, *J. biol. Chem.* **207,** 245 (1954).
10. O. O. Blumenfeld and G. E. Perlmann, *J. biol. Chem.* **236,** 2472 (1961).