The effect of -SH reagents on the activity of ribonuclease

In a systematic study of a large number of enzymes it was found that ribonuclease belonged to the group of non -SH enzymes[1]. As many of the reagents used for the destruction of the -SH groups combine as well with other groups in the protein molecule, such as OH, NH_2, and carboxyl groups, it was emphasized: (1) that tests for -SH enzymes should be made with the three types of reagents--oxidizing, mercaptide-forming, and alkylating reagents; (2) that inhibition with oxidizing or with mercaptide-forming reagents should be restored on addition of thiols (cysteine, glutathion, dimercaptopropanol); (3) that contact between reagent and enzyme should be limited to a short time (a few minutes) to avoid action on other groups. Moreover, it was pointed out that only the -SH groups of the *native* protein were essential for enzyme activity in the -SH enzymes.

A number of papers have been published by LEDOUX[2,3,4] maintaining that (1) ribonuclease activity is inhibited by -SH reagents, and (2) that ribonuclease is an -SH enzyme. Because of LEDOUX's publications it was decided to reinvestigate this problem.

Recrystallized ribonuclease was obtained from Worthington Biochemical Laboratory and from Armour and Company. Both gave identical results. Activity of the enzyme was determined manometrically at pH 7.6 and 18°. The inhibitors were added to the enzyme solution, and after ten minutes ribonuclease (Schwartz purified yeast ribonucleic acid) was added from the side arm. None of the usual reagents for testing the activity of -SH enzymes had any effect at all on the activity of ribonuclease. The ratio of inhibitor:enzyme varied from 356:1 to 4,270:1 (Table I).

TABLE I

EFFECT OF -SH REAGENTS ON ACTIVITY OF RIBONUCLEASE

Ribonuclease, 0.4 ml (40 micrograms); inhibitor, 0.3 ml; NaCl-bicarbonate buffer, 2.0 ml. Yeast ribonucleate, 6 mg in 0.3 ml. Gas phase, N_2:CO_2; pH 7.6; temp., 18°; time, 15 min.

Inhibitor	*Concentration M*	*CO_2 Formation µl*	*Ratio Inhibitor: Enzyme*
None		45.0	
Iodoacetamide	$3.6 \cdot 10^{-3}$	46.0	4,270
Iodosobenzoate	$3.4 \cdot 10^{-3}$	43.0	4,030
p-Cl-Hg benzoate	$1 \cdot 10^{-3}$	47.0	3,560
N-ethylmaleimide	$3.6 \cdot 10^{-3}$	46.0	4,270
Mersalyl	$3.6 \cdot 10^{-4}$	47.0	427
p-Carboxyphenylarsine oxide	$1 \cdot 10^{-4}$	45.0	356
Oxidized glutathione	$1 \cdot 15^{-4}$	46.0	356

LEDOUX[4] maintains that ribonuclease contains four -SH groups per mole. We have again tested ribonuclease for -SH groups— by oxidation with ferricyanide, with iodosobenzoate, and by amperometric titration. In no case were any -SH groups detected. These negative results agree with those of ANFINSEN *et al.*[5]. According to them, ribonuclease consists of a single chain arranged in a compact, folded structure, crosslinked through four disulfide bridges.

TABLE II

TITRATION OF RIBONUCLEASE WITH IODOSOBENZOATE

Enzyme, 25 mg, in 5 ml of 0.1 *M* phosphate, pH 7.0. Titration of iodosobenzoate three minutes after addition of reagent to protein.

Iodosobenzoate M	*Ratio Iodosobenzoate: enzyme*	*Iodosobenzoate utilized*
$2.5 \cdot 10^{-7}$	1.58	None
$5.0 \cdot 10^{-7}$	3.16	None
$10.0 \cdot 10^{-7}$	6.32	$8.25 \cdot 10^{-8}$
$20 \cdot 10^{-7}$	12.64	$1.3 \cdot 10^{-7}$
$200 \cdot 10^{-7}$	126.4	$17 \cdot 10^{-7}$

The reason for the disagreement with LEDOUX must be looked for in the excessively large amounts of inhibitor and the long incubation periods used in his experiments. Under those conditions all the reagents, with the exception of ethylmaleimide, combine with amino groups. With iodosobenzoate, for example, at a ratio of reagent:enzyme of 100,000:1 he required an incubation of 30 minutes at pH 7 to produce an inhibition of 30%[3]. As emphasized by HELLERMAN *et al.*[6] iodosobenzoate is a reliable –SH reagent *only* when a slight excess is used. On addition of increasing amounts of iodosobenzoate, the utilization by the protein increased, an indication that other groups, such as OH and NH_2 groups, were oxidized (Table II). With H_2O_2 LEDOUX had to increase the ratio to two million to one. It is known[7,8] that H_2O_2 produces deamination of amino acids when used in excess. On addition of $1 \cdot 10^{-4}$ *M* H_2O_2 and $5 \cdot 10^{-5}$ *M* $FeSO_4$, to $1 \cdot 10^{-4}$ *M* amino acid, there was deamination of histidine (50%), tryptophan (50%), and tyrosine (38%)[9].

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Chemical Division, Department of Medicine, The University of Chicago, Chicago, Illinois (U.S.A.)

M. RABINOVITCH*
E. S. GUZMAN BARRON

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A fluorescent derivative of guanine formed during the hydrolysis of DNA

LEVY AND SNELLBAKER[1] have described a fluorescent compound (W.S.), which they have isolated from DNA's from several sources and have suggested it might be a naturally occurring base. Evidence presented below shows that W.S. is a product formed from guanine during the hydrolysis of the nucleic acid.

It was confirmed that the hydrolysis products of thymus DNA formed by the action of *N* HCl for 1 hour at 100° contained a compound with the properties of W.S. This was separated from the other products by 2 dimensional chromatography in *iso*propanol-water-hydrochloric acid[2] and *iso*propanol–water–ammonia[3]. (W.S. has the same R_F as cytosine in the first solvent and a slightly smaller R_F than cytosine in the second.). For estimation, cytosine and W.S. were eluted simultaneously; portions of the eluate were concentrated and W.S. and cytosine were separated by paper electrophoresis at pH 3.5. At this pH in ammonium formate buffer, W.S. remained stationary (*cf.* ref. 1) and cytosine moved as a cation. As shown in the table, the absorption at 258 mμ of the W.S. formed by the hydrolysis was equal to 17% of the absorption at 250 mμ of the liberated guanine. Hydrolysis with *N* HCl at 55° for 1 hour, which releases the purines guanine, adenine and 6-methyl-aminopurine[4], did not release any W.S. from thymus DNA. Apurinic acid prepared by this hydrolysis was dialysed against 0.1 *N* HCl and then against water. After hydrolysis in *N* HCl at 100° and separation on the same solvents, apurinic acid showed only 4% of the amount of W.S. present in an equivalent amount of DNA hydrolysed in the same way. The apurinic acid was found to contain residual amounts of purines sufficient to account for the presence of the small amount of W.S.

The properties of W.S.: fluorescence, $E_{max.}$ at pH 1 at 258 mμ, insolubility in alkaline solvents, p*K* values[1], suggested it might be derived from guanine. When 4 mg guanine were added to 4 mg of apurinic acid and the mixture hydrolysed with *N* HCl at 100°, the amount of W.S. formed was greatly increased, as shown in the table. Less guanine was obtained by *N* HCl hydrolysis at 100° of apurinic acid and thymus DNA, than was obtained by 72% perchloric acid hydrolysis. The difference was approximately proportional to the amount of W.S. formed. Adenine did not show any corresponding difference, as shown below.

In agreement with LEVY AND SNELLBAKER, I found guanine deoxyriboside, hydrolysed with