Biochemical Pharmacology, Vol. 17, pp. 769–777. Pergamon Press. 1968. Printed in Great Britain

REACTION OF THE CARCINOGEN 4-HYDROXYAMINOQUINOLINE 1-OXIDE WITH SULFHYDRYL GROUPS OF PROTEINS

MOTOO HOZUMI*

Orchard Park Laboratories, Roswell Park Memorial Institute, Orchard Park, N.Y., U.S.A.

(*Received* 15 *August* 1967; *accepted* 30 *October* 1967)

Abstract—Reaction of 4-hydroxyaminoquinoline 1-oxide (4HAQO) with sulfhydryl groups of albumin and enzymes with different types of sulfhydryl groups was studied by direct determination of unreacted sulfhydryl groups and by measurement of enzyme activities.

Sulfhydryl groups of albumin, catalase, and alcohol dehydrogenase were markedly consumed in the presence of 4HAQO, and the consumption was clearly dependent on the concentration of 4HAQO. The activities of catalase, alcohol dehydrogenase, and urease were inhibited in the presence of 4HAQO, but the activity of pancreatic lipase was not affected by 4HAQO. The inhibition of these enzymes with similar molar ratios of 4HAQO to sulfhydryl groups varied as follows: catalase > alcohol dehydrogenase > urease.

Inhibition of alcohol dehydrogenase and urease was almost completely reversed by the addition of glutathione, but the effect of glutathione on the inhibition of catalase was not studied. The insensitivity of lipase to 4HAQO was probably due to inaccessibility of the sulfhydryl groups to 4HAQO, for the enzyme was inhibited markedly by *p*-chloromercuribenzoate, but not by iodosobenzoate under the same experimental conditions. The different sensitivities of sulfhydryl groups in other enzymes to 4HAQO appear to be related to the differences in the locations of the sulfhydryl groups in the enzyme molecules. The reactions of sulfhydryl groups of proteins with 4HAQO may be significant in the mechanism of 4HAQO carcinogenesis.

THE SULPHYDRYL groups of glutathione and cysteine were recently found to be readily oxidized *in vitro* at pH 7·0 at 37° by 4-hydroxyaminoquinoline 1-oxide (4HAQO), an apparent proximate carcinogenic metabolite of 4-nitroquinoline 1-oxide (4NQO), but not by 4-aminoquinoline 1-oxide (4AQO), a noncarcinogenic metabolite of 4NQO.[1–3] Study of the mechanism of the oxidation of the sulfhydryl groups showed that the oxidation occurred through the catalytic action of 4HAQO in the presence of oxygen, without any detectable change in the 4HAQO. Thus the mechanism was entirely different from the substitution reaction between 4NQO and sulfhydryl compounds.[3]

Although sulfhydryl groups in small molecules such as glutathione and cysteine were easily oxidized in the presence of 4HAQO, it remained unclear whether the sulfhydryl groups of macromolecular proteins could be oxidized by 4HAQO. A study of the effects of 4HAQO on proteins can be expected to shed light on the mechanism of carcinogenesis induced by quinoline derivatives, since proteins play a dominant

* Present address: National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo, Japan.

role in cellular metabolism[4–9] and protein integrity may play an important role in carcinogenesis,[10–14] and since 4HAQO is believed to be a proximate carcinogenic metabolite of 4NQO.[1, 2]

MATERIALS AND METHODS

Reagents. Crystalline yeast alcohol dehydrogenase (alcohol:NAD oxidoreductase), crystalline jack bean urease (urease amidohydrolase), crystalline beef liver catalase (H_2O_2:H_2O_2 oxidoreductase), crude pancreatic lipase (glycerol ester hydrolase, "steapsin"), and crystalline bovine serum albumin were obtained from the Sigma Chemical Co.

4HAQO was prepared by reducing 4NQO with phenylhydrazine according to the method of Ochiai and Mitarashi.[15] Spectroanalyzed dimethyl sulfoxide (DMSO) was obtained from the Fisher Scientific Co. Only trace contaminants in the solvent could be detected by gas chromatographic analysis with a flame ionization detector.

Thin-layer chromatography (TLC). TLC was carried out, on the basis of a recent report by Sugimura *et al.*[16] that 4HAQO and its derivatives could be separated in this way, by using Eastman silica gel without fluorescent indicator (Distillation Products Industries, Rochester, N.Y.) as the supporting material, and the upper layer of a 1:1:1 mixture of ethyl acetate–*sec*-butanol–water as the developing solvent system. Time for development was 3 hr at room temperature.

Assay of enzymatic activities. The enzymatic activities of alcohol dehydrogenase, catalase, urease, and pancreatic lipase were determined by the methods of Racker,[17] Fujimoto,[18] Sumner,[19] and Bier[20] respectively. Because 4HAQO is almost insoluble in water, stock solutions were prepared in DMSO, which has excellent solvent properties and is presumably harmless to tissues and enzymes.[21–24] The stock solutions were diluted with water to provide concentrations such that 2 ml would contain the desired amount of 4HAQO. The final concentrations of DMSO in the reaction mixtures were between 0·5 and 2%. Each sample contained 100 μg alcohol dehydrogenase, 40 μg catalase, 54 μg urease or 2 mg lipase, dissolved in 2 ml of 0·05 M (pH 7·0) phosphate buffer, and was incubated with 2 ml of 4HAQO solution in a water bath at 37° for 1 hr. After reaction, aliquots of the incubation mixtures were used for the determination of enzymatic activities.

Determination of sulfhydryl groups. Reactions of sulfhydryl groups with 4HAQO were studied chemically for albumin and enzymes that could be obtained in quantity in the pure state. The reactions were carried out for 1 hr in a water bath at 37°. For albumin, 4 ml of a solution containing 55·2 mg of the protein and 4 ml of an aqueous solution containing the appropriate amount of the stock DMSO solution of 4HAQO were incubated with 1 ml of 10^{-2} M EDTA and 1 ml of 10^{-1} M (pH 7·0) phosphate buffer. For the enzymes, 2 ml of 0·05 M (pH 7·0) phosphate buffer containing 5 mg alcohol dehydrogenase or 10 mg catalase was incubated with 2 ml of 4HAQO solution in the absence of EDTA. After the 1-hr incubation period, aliquots of the incubation mixture were stirred for 30 min with 30 ml of pH 7·4 Tris–HNO_3 buffer containing 10 M sodium lauryl sulfate; then the unconsumed sulfhydryl groups were determined with an amperometer according to the method of Benesch *et al.*[25] Sodium lauryl sulfate was used because detergents are excellent denaturing compounds for protein,[26] yielding more consistent and reproducible determinations of sulfhydryl groups.

RESULTS

Physicochemical properties of 4HAQO in DMSO solution. 4HAQO was far more soluble in DMSO than in any other conventional organic solvent such as methanol, ethanol, acetone, benzene or *p*-dioxane. With vigorous shaking and heating at approximately 60°, a 1×10^{-1} M 4HAQO solution was obtained with DMSO, whereas, at most, a 1×10^{-3} M solution was obtained with other organic solvents. In order to see whether any interaction occurs between 4HAQO and DMSO, some physicochemical characteristics of 4HAQO in DMSO were studied, with methanol as a control solvent. The u.v. absorption spectrum of 4HAQO in DMSO was observed by first dissolving 4HAQO in DMSO and then diluting with methanol to give a 1% DMSO solution of 4HAQO in methanol. A 1% solution of DMSO in methanol was transparent from 240–400 mμ, and the absorption spectrum of the DMSO solution of 4HAQO in methanol, with maxima at 243, 260 and 369 mμ, was identical with that of 4HAQO in absolute methanol (Fig. 1). In TLC, the R_f values of various concentrations of 4HAQO in DMSO were identical with those of the corresponding concentrations in absolute methanol.

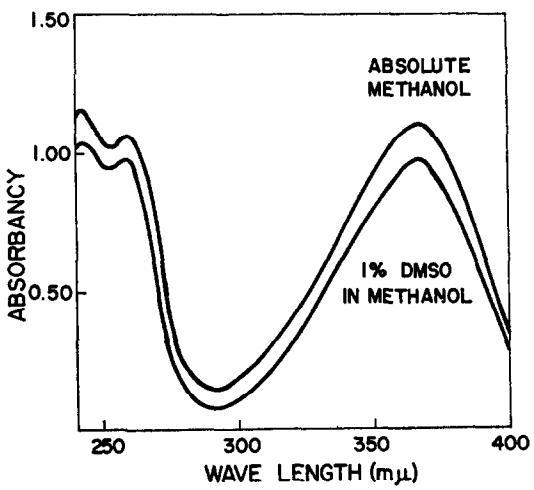

FIG. 1. Ultraviolet absorption spectra of 4HAQO in DMSO and methanol solutions. First 4HAQO was dissolved in DMSO, and then the solution was diluted with methanol to give a 1% DMSO solution of 4HAQO in methanol. The concentration of 4HAQO both in absolute methanol and in 1% DMSO in methanol was 0·08 μmole/ml. Absorption spectra of 4HAQO solutions were measured with a Cary recording spectrophotometer.

Consumption of sulfhydryl groups of proteins by 4HAQO. The reactions of 4HAQO with the sulfhydryl groups of bovine serum albumin, yeast alcohol dehydrogenase and beef liver catalase were studied chemically. The sulfhydryl groups of all of these proteins were markedly consumed by 4HAQO (pH 7·0) at 37° either in the presence (albumin) or absence (alcohol dehydrogenase and catalase) of EDTA. The percentages of consumption were clearly dependent on the concentration of 4HAQO (Table 1). During the incubation period, no consumption of sulfhydryl groups was observed without 4HAQO in either the presence or the absence of EDTA. The sulfhydryl groups of catalase were the most sensitive to 4HAQO, being consumed 85 and 94 per cent at the respective molar ratios of 1·4 and 2·8 4HAQO/sulfhydryl. The sulfhydryl

groups of albumin and alcohol dehydrogenase were consumed 100 and 79 per cent at the respective molar ratios of 9·0 and 5·0.

Inhibition of sulfhydryl enzyme activities by 4HAQO. The effects of 4HAQO on the activities of various sulfhydryl enzymes, including alcohol dehydrogenase and catalase, were observed under the same experimental conditions as those under which

TABLE 1. PERCENTAGE OF SULFHYDRYL GROUPS CONSUMED DURING INCUBATION OF SULFHYDRYL COMPOUNDS WITH 4HAQO*

Sulfhydryl compounds	Concentration of		Molar ratio of 4 HAQO to SH†	Sulfhydryl groups consumed (%)
	Sulfhydryl compounds (μg/ml)	4HAQO (M)		
Albumin	5520	0	0	0‡
		1×10^{-4}	1·8	37
		5×10^{-4}	9·0	100
Alcohol dehydrogenase	1250	0	0	0‡
		5×10^{-5}	0·5	25
		5×10^{-4}	5·0	79
Catalase	2500	0	0	0‡
		$6{\cdot}25 \times 10^{-5}$	0·7	26
		$1{\cdot}25 \times 10^{-4}$	1·4	85
		$2{\cdot}5 \times 10^{-4}$	2·8	94

* Sulfhydryl compounds were incubated with 4HAQO for 1 hr at 37° in 10 μmole/ml phosphate buffer, pH 7·0, in the presence of 1 μmole/ml EDTA (for albumin) or in 50 μmole/ml pH 7·0 phosphate buffer without EDTA (for alcohol dehydrogenase or catalase).

† Sulfhydryl groups per mole of protein were as follows: albumin (mol. wt. 69,000[27]), 0·68; alcohol dehydrogenase (mol. wt. 150,000[28]), 12; catalase (mol. wt. 248,000[29]), 8·4.

‡ Control values for μmoles of sulfhydryl groups in 1 ml incubation mixture were as follows: albumin, 0·027; alcohol dehydrogenase, 0·040; catalase, 0·034.

the reaction of 4HAQO and the sulfhydryl groups of proteins was observed. 4HAQO markedly inhibited all of the enzymes except lipase, and the inhibition was clearly dependent on the concentration of 4HAQO (Table 2). The inhibition of alcohol dehydrogenase and catalase at the comparatively low respective molar ratios of 0·5 and 0·7 4HAQO/sulfhydryl was closely correlated with the chemical consumption of sulfhydryl groups shown in Table 1. At the respective molar ratios of 5 and 1·4 4HAQO/sulfhydryl for the same two enzymes, however, there was significantly less inhibition of enzyme activity than chemically determined loss of sulfhydryl groups. Inhibition of urease was generally less than inhibition of alcohol dehydrogenase and catalase at similar molar ratios of 4HAQO to sulfhydryl; differences among these enzymes were greater at higher molar ratios. Lipase was not inhibited, even at 5×10^{-3} M 4HAQO.

DMSO at concentrations from 0·5 to 2% slightly affected the activities of the enzymes under the experimental conditions utilized. The activities of alcohol dehydrogenase and urease decreased 5 and 4 per cent, respectively, in 1% DMSO, whereas the activity of catalase increased 7 per cent in 0·5% DMSO. Lipase activity did not change in 2% DMSO. Thus the action of DMSO, if any, did not affect the enzyme assays to any important extent.

Effects of glutathione on the inhibition of alcohol dehydrogenase and urease by 4HAQO. Glutathione counteracted almost completely the inhibition of alcohol

dehydrogenase and urease when added simultaneously with 4HAQO (Table 3). Inhibition of alcohol dehydrogenase was reversed almost completely 10 min after the enzyme–4HAQO mixture was incubated with glutathione.

TABLE 2. PERCENTAGE OF ENZYMATIC ACTIVITY INHIBITED AFTER INCUBATION OF SULFHYDRYL ENZYMES WITH 4HAQO*

Sulfhydryl enzymes	Concentration of: Sulfhydryl enzymes (μg/ml)	Concentration of: 4HAQO (M)	Molar ratio of 4 HAQO to SH†	Enzyme activity inhibited (%)
Alcohol dehydrogenase	25	0	0	0‡
		1×10^{-6}	0·5	12
		1×10^{-5}	5	17
		1×10^{-4}	50	31
		1×10^{-3}	500	74
Catalase	10	0	0	0‡
		$2{\cdot}5 \times 10^{-7}$	0·7	24
		5×10^{-7}	1·4	51
		1×10^{-6}	2·8	89
Urease	13·5	0	0	0‡
		1×10^{-5}	7·7	20
		1×10^{-4}	77	33
		1×10^{-3}	777	50
Lipase	500	0	—	0
		1×10^{-3}	—	−4
		5×10^{-3}	—	2

* Sulfhydryl enzymes were incubated with 4HAQO for 1 hr at 37° in 50 μmole/ml pH 7·0 phosphate buffer. Enzymatic activities were determined with aliquots of the reaction mixture as described (Materials and Methods).

† Sulfhydryl groups per mole of protein were as follows: alcohol dehydrogenase (mol. wt. 150,000[28]), 12; catalase (mol. wt. 248,000[29]), 8·4; urease (mol. wt. 473,000[30]), 46[31]; lipase (mol. wt. uncertain).

‡ Control values were as follows: catalase, K = 20·6/mg/min.; alcohol dehydrogenase, reduction of 108 μmole/NAD/mg/min; urease, release of 44·1 mg nitrogen/mg/min (average during 5 min); lipase, release of 20 μmole acid/mg/hr.

Effects of p-chloromercuribenzoate and iodosobenzoic acid on pancreatic lipase in comparison with those of 4HAQO. Pancreatic lipase was not inhibited by 4HAQO (Table 4). Wills[32] reported that the enzyme was inhibited by *p*-chloromercuribenzoate (PCMB) and iodine, but not by other sulfhydryl reagents such as ferricyanide, iodosobenzoic acid and iodoacetate. Accordingly, the effects of PCMB, iodosobenzoic acid and 4HAQO on pancreatic lipase were measured, under the usual experimental conditions of the present study, to ascertain whether the lack of effect of 4HAQO on the enzyme might be due to a unique preparation of the enzyme or to experimental conditions. Under identical conditions, the lipase was markedly inhibited by PCMB but not at all by iodosobenzoic acid or 4HAQO, even when the latter two reagents were employed at much higher concentrations than that of PCMB.

DISCUSSION

A definite consumption of the sulfhydryl groups of albumin, alcohol dehydrogenase, and catalase by 4HAQO was observed under the same conditions (pH 7·0 and 37°) employed previously for the oxidation of glutathione and cysteine.[3] Nevertheless, the extent of reaction of these protein sulfhydryl groups was significantly lower than

TABLE 3. EFFECT OF GLUTATHIONE ON THE INHIBITION OF ALCOHOL DEHYDROGENASE AND UREASE BY 4HAQO*

Concentration of 4HAQO (M)	Glutathione added (M)	Enzyme activity inhibited (%)	
		Alcohol dehydrogenase (25 μg/ml)	Urease (13·5 μg/ml)
0	0	0†	0†
0	$2{\cdot}5 \times 10^{-3}$	—	−4
0	5×10^{-3}	−5	—
0	$2{\cdot}5 \times 10^{-2}$	—	2
0	5×10^{-2}	−5	—
$1{\cdot}7 \times 10^{-5}$	0	17	—
$1{\cdot}7 \times 10^{-5}$	5×10^{-3}	0	—
5×10^{-5}	0	—	17
5×10^{-5}	$2{\cdot}5 \times 10^{-3}$	—	2
$1{\cdot}7 \times 10^{-4}$	0	38	—
$1{\cdot}7 \times 10^{-4}$	5×10^{-3}	−4	—
$1{\cdot}7 \times 10^{-4}$	5×10^{-2}	−3	—
$1{\cdot}7 \times 10^{-4}$	5×10^{-2}‡	3	—
5×10^{-4}	0	—	38
5×10^{-4}	$2{\cdot}5 \times 10^{-3}$	—	4
5×10^{-4}	$2{\cdot}5 \times 10^{-2}$	—	5

* Sulfhydryl enzymes were incubated with 4HAQO for 1 hr at 37° in 50 μmole/ml pH 7·0 phosphate buffer; glutathione was added at the beginning of the hour, except where otherwise indicated. Total volume was 3 ml. Enzymatic activities were determined with aliquots of the reaction mixtures as described (Materials and Methods).

† Control values were as follows: alcohol dehydrogenase, reduction of 110 μmole NAD/mg/min; urease, release of 44 mg nitrogen/mg/min (average during 5 min).

‡ Glutathione was added after the reaction mixture had been incubated for 1 hr at 37° and had stood for 10 min at room temperature.

TABLE 4. EFFECTS OF PCMB AND IODOSOBENZOIC ACID ON PANCREATIC LIPASE*

Sulfhydryl inhibitors	Concentration (M)	Lipase activity inhibited (%)
None	—	0†
PCMB	2×10^{-4}	34
PCMB	1×10^{-3}	64
Iodosobenzoic acid	1×10^{-3}	2
Iodosobenzoic acid	2×10^{-3}	−8
4HAQO	5×10^{-3}	−3

* Sulfhydryl inhibitors were incubated with 500 μg/ml lipase for 1 hr at 37° in 50 μmole/ml pH 7·0 phosphate buffer. Total volume was 4 ml. Lipase activities were determined with aliquots of the reaction mixtures as described (Materials and Methods).

† The control value was the release of 22 μmole acid/mg/hr.

the reaction of glutathione and cysteine at the same molar ratio of 4HAQO to sulfhydryl groups. The sulfhydryl groups of glutathione and cysteine were consumed completely at a molar ratio of 1 4HAQO/sulfhydryl at pH 7·0 and 37° for 1 hr, whereas 5–9 times as much 4HAQO was required for the complete consumption of the sulfhydryl groups of proteins under the same conditions.

The correlation between the inhibition of enzyme activity and the consumption of

sulfhydryl groups in some of these enzymes by 4HAQO indicates that 4HAQO reacts with sulfhydryl groups that are essential for enzyme activity. The ability of glutathione to reverse the inhibition of these enzymes by 4HAQO is further evidence that the inhibition was due to loss of the sulfhydryl groups.

Sulfhydryl groups in lipase were not determined because the preparation of lipase was crude and was assumed to be unsuitable for exact determination of the sulfhydryl groups of the lipase itself, and those in urease were not determined because a sample of urease large enough for determination of sulfhydryl groups was not available. On the other hand, the effect of glutathione on the inhibition of catalase by 4HAQO was not observed because the activity of catalase is known to be inhibited by various sulfhydryl compounds,[33-35] even though catalase contains sulfhydryl groups responsible for enzyme activity.[36, 37]

Although inhibition of the enzyme activities of alcohol dehydrogenase and catalase at relatively low levels of 4HAQO was closely correlated with sulfhydryl consumption, enzyme inhibition at higher 4HAQO levels was significantly less than sulfhydryl consumption. This difference may be due to reaction between 4HAQO and sulfhydryl groups during the 30-min treatment of the incubation mixtures with detergent prior to titration of the sulfhydryl groups, the reaction being perhaps more pronounced at high 4HAQO levels. Alternatively, some of the sulfhydryl groups in the enzyme preparations may not be directly concerned with enzyme activity. If these "nonenzymatic" sulfhydryl groups are chemically less active, they would be attacked only at relatively high 4HAQO levels.

At similar molar ratios of 4HAQO to sulfhydryl groups, the consumption of sulfhydryl groups and the inhibition of enzyme activity were greater for catalase than for alcohol dehydrogenase. Inhibition of activity was generally less for urease than for alcohol dehydrogenase. Lipase was not inhibited at all by 4HAQO, although it was inhibited markedly by PCMB. The different sensitivities of these various enzymes to 4HAQO may be attributed to the presence of two or more different types of sulfhydryl groups in the molecules of the enzymes.

The nature of the sulfhydryl groups in catalase has not been demonstrated clearly, but several experiments show that the sulfhydryl groups in this enzyme are necessary for its activity.[36, 37] Alcohol dehydrogenase contains a maximum of 36 sulfhydryl groups per mole of enzyme, all of which are freely reacting and necessary for enzyme activity.[28, 38] On the other hand, urease contains equal amounts of two different types of sulfhydryl groups in the same molecule.[31] Thus 23 sulfhydryl groups per mole of urease react with dilute ferricyanide, dilute iodosobenzoate, or porphyrindin without a concomitant loss of enzymatic activity. Further oxidation of the sulfhydryl groups with iodine or high concentrations of iodosobenzoate, or reaction with PCMB reveals the presence of an additional 23 sulfhydryl groups per mole of urease, the oxidation of which results in complete inactivation of the enzyme. The lower sensitivity of urease than of alcohol dehydrogenase to 4HAQO may be due to the presence of the latter relatively unreactive sulfhydryl groups in urease. Since the sulfhydryl groups in urease unrelated to enzymatic activity are similar in nature to those in alcohol dehydrogenase, they are presumably oxidized likewise by 4HAQO.

Wills[32] found that purified pancreatic lipase was entirely unaffected by reagents that generally react with freely reacting sulfhydryl groups, but it was markedly inhibited by iodine or PCMB. Accordingly, the sulfhydryl groups in lipase were assumed to be

the sluggish type, located inside the enzyme molecule.[32] In the present study, lipase was also inhibited by PCMB but not by iodosobenzoate, as reported by Wills, and it was entirely resistant to 4HAQO. Since Wills used purified lipase in his experiments and observed the effects of sulfhydryl reagents on this enzyme, as has been mentioned, the insensitivity of lipase to 4HAQO is probably another manifestation of a type of sulfhydryl group that is unreactive to mild reagents.

Although the reduced form of NAD has been found to be oxidized by 4HAQO,* almost no oxidation of NAD was observed during the determination of alcohol dehydrogenase activity in the present study, and there was no difficulty in the measurements.

This study shows that 4HAQO reacts with various types of protein sulfhydryl groups, although the reactivity is dependent on the nature or location of the sulfhydryl groups in the protein molecules. Marked reaction of sulfhydryl groups in various cellular proteins may significantly change the metabolism of cells. Some of these metabolic disturbances may be responsible for the induction of tumors by 4HAQO. Although direct interactions between DNA and 4HAQO, which were recently observed in various systems *in vitro*,[39–45] may also play important roles in 4HAQO carcinogenesis, it has been suggested that an interaction of a carcinogen and a certain cellular protein could induce a permanently changed and stable metabolic situation without the necessity of any direct interaction of the carcinogen and genetic material. Such cells in the permanently altered metabolic situation may be destined to become neoplastic.[11]

It was demonstrated in the present study that DMSO not only has outstanding solvent property for 4HAQO without apparently affecting the properties of the solute, but also can be present in solutions of various sulfhydryl enzymes without causing any significant effects corresponding to those reported for many other enzymes.[22–24] It thus promises to be an excellent solvent for further biochemical studies of the mechanism of carcinogenesis induced by 4HAQO.

Acknowledgement—The author is indebted to Drs. F. G. Bock and R. J. Shamberger for their critical reading of this manuscript.

REFERENCES

1. H. ENDO and F. KUME, *Gann* **56**, 261 (1965).
2. Y. SHIRASU, *Proc. Soc. exp. Biol. Med.* **118**, 812 (1965).
3. M. HOZUMI, S. INUZUKA and T. SUGIMURA, *Cancer Res.* **27**, 1378 (1967).
4. E. S. G. BARRON, *Adv. Enzymol.* **11**, 201 (1951).
5. R. CECIL, in *The Proteins* (Ed. H. NEURATH), vol. 1, 2nd edn. p. 379. Academic Press, New York and London (1963).
6. J. L. WEBB, *Enzymes and Metabolic Inhibitors*, vol. 2, p. 635. Academic Press, New York and London (1966).
7. P. D. BOYER, in *The Enzymes* (Eds. P. D. BOYER, H. LARDY and K. MYRBÄCK), vol. 1, 2nd edn, p. 511. Academic Press, New York and London (1965).
8. L. G. EGYÜD and A. SZENT-GYÖRGYI, *Proc. natn. Acad. Sci. U.S.A.* **55**, 388 (1966).
9. T. TAMAOKI and F. MIYAZAWA, *J. molec. Biol.* **23**, 35 (1967).
10. P. RONDONI, *Adv. Cancer Res.* **3**, 171 (1955).
11. H. C. PITOT and C. HEIDELBERGER, *Cancer Res.* **23**, 1694 (1963).
12. C. Heidelberger, *J. cell. comp. Physiol.* **64**, *suppl.* 1, 129 (1964).
13. J. A. MILLER and E. C. MILLER, *Lab. Invest.* **15**, 217 (1966).
14. E. C. MILLER and J. A. MILLER, *Pharmac. Rev.* **18**, 805 (1966).

* T. Sugimura, private communication.

15. E. OCHIAI and H. MITARASHI, *Rep. ITSUU Lab.* **13**, 19 (1963).
16. T. SUGIMURA, K. OKABE and H. ENDO, *Gann* **56**, 489 (1965).
17. E. RACKER, *J. biol. Chem.* **184**, 313 (1950).
18. S. FUJIMOTO, *Cancer Res.* **25**, 534 (1965).
19. J. B. SUMNER, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. 2, p. 379. Academic Press, New York (1955).
20. M. BIER, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. 1, p. 627. Academic Press, New York (1955).
21. T. INAGAMI and J. M. STURTEVANT, *Biochim. biophys. Acta* **38**, 64 (1960).
22. K. HAMAGUCHI, *J. Biochem., Tokyo*, **56**, 441 (1964).
23. D. H. RAMMLER and A. ZAFFARONI, *Ann. N.Y. Acad. Sci.* **141**, 13 (1967).
24. D. H. RAMMLER, *Ann. N.Y. Acad. Sci.* **141**, 291 (1967).
25. R. E. BENESCH, H. A. LARDY and R. BENESCH, *J. biol. Chem.* **216**, 663 (1955).
26. M. JOLY, *A Physico-chemical Approach to the Denaturation of Proteins*, p. 30. Academic Press, New York and London (1965).
27. E. J. COHN, W. L. HUGHES, JR. and J. H. WEARE, *J. Am. chem. Soc.* **69**, 1753 (1947).
28. J. E. HAYES and S. F. VELICK, *J. biol. Chem.* **207**, 225 (1954).
29. J. B. SUMNER and N. GRALÉN, *J. biol. Chem.* **125**, 33 (1938).
30. J. B. SUMNER, N. GRALÉN and I.-B. ERIKSSON-QUENSEL, *J. biol. Chem.* **125**, 37 (1938).
31. L. HELLERMAN, F. P. CHINARD and V. R. DEITZ, *J. biol. Chem.* **147**, 443 (1943).
32. E. D. WILLS, *Biochim. biophys. Acta* **40**, 481 (1960).
33. F. ABRIGNANI and V. MUTOLO, *Boll. Soc. ital. Biol. sper.* **31**, 226 (1955).
34. G. CERIOTTI, L. SPANDRIO and E. BERTI, *Biochim. biophys. Acta* **23**, 362 (1957).
35. H. HIRAI and H. F. DEUTSCH, *Cancer Res.* **18**, 283 (1958).
36. E. S. COOK, C. W. KREKE, M. L. MCDEVITT and M. D. BARTLETT, *J. biol. Chem.* **162**, 43 (1946).
37. C. R. HEISLER and H. Y. YANG, *Biochem. biophys. Res. Commun.* **23**, 660 (1966).
38. E. S. G. BARRON and S. LEVINE, *Archs Biochem. Biophys.* **41**, 175 (1952).
39. T. OKABAYASHI, *Chem. pharm. Bull., Tokyo* **10**, 1127 (1962).
40. T. ONO and M. OHASHI, *Proc. Jap. Cancer Ass. 22nd gen. Meet., Tokyo*, p. 191 (1963).
41. T. OKABAYASHI, A. YOSHIMOTO and M. IDE, *Chem. pharm. Bull., Tokyo* **12**, 257 (1964).
42. T. OKABAYASHI, M. IDE, A. YOSHIMOTO and M. OTSUBO, *Chem. pharm. Bull.*, Tokyo **13**, 610 (1965).
43. C. NAGATA, M. KODAMA, Y. TAGASHIRA and A. IMAMURA, *Biopolymers* **4**, 409 (1966).
44. M. F. MALKIN and A. C. ZAHALSKY, *Science* **154**, 1665 (1966).
45. M. ISHIZAWA and H. ENDO, *Biochem. Pharmac.* **16**, 637 (1967).