Toxic effects of ozone on murine L929 fibroblasts

Enzyme inactivation and glutathione depletion

Jolanda VAN DER ZEE,* Tom M. A. R. DUBBELMAN,* Ton K. RAAP† and Johnny VAN STEVENINCK*‡

*Department of Medical Biochemistry and †Department of Histochemistry and Cytochemistry, University of Leiden, Sylvius Laboratories, P.O. Box 9503, 2300 RA Leiden, The Netherlands

Exposure of L929 murine fibroblasts to ozone resulted in K⁺ leakage and inhibition of several enzymes. Most sensitive to ozone exposure were glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase. The activities of another cytosolic enzyme, lactate dehydrogenase, the mitochondrial enzymes glutamate dehydrogenase, succinate dehydrogenase, cytochrome *c* oxidase and the activity of the lysosomal enzymes acid phosphatase and β -glucuronidase were, initially, not or only slightly affected. The localization of the lysosomal enzymes did not change during ozone exposure. After prolonged exposure complete deterioration of the cells was observed and all enzyme activities declined. The activity of the enzymes was also monitored during ozone exposure of a sonicated cell suspension and it was shown that all these enzymes are in fact susceptible to ozone. These observations clearly demonstrate that, besides the structure and amino acid composition of an enzyme, the localization in the cell plays an important role in its susceptibility to ozone. The intracellular levels of reduced and oxidized glutathione were affected as well. The ATP content, however, proved to be insensitive to ozone exposure.

INTRODUCTION

Ozone, an important component of photochemical air pollution, is a very powerful oxidant. The molecular targets for reaction with ozone include most cellular components (Menzel, 1976, 1984). In aqueous solution, cysteine, methionine, tryptophan, tyrosine, histidine and phenylalanine are oxidized by ozone (Mudd *et al.*, 1969). Ozone also readily reacts with thiols and amino acids in tissue protein. Furthermore, oxidation of unsaturated fatty acids by ozone has been shown, resulting in lipid peroxidation (Menzel, 1976). In other studies the reactivity of ozone with nucleic acids and derivatives has been investigated (Shinriki *et al.*, 1984).

Several observations suggest that tissue and cell damage results from oxidation of membrane constituents (Pauls & Thompson, 1981; Morgan & Wenzel, 1985). There is evidence, however, that the effects of ozone extend beyond the plasma membrane. *In vitro* studies of erythrocytes showed that GAPDH, an enzyme attached to the cytoplasmic side of the membrane, was inhibited and that the intracellular glutathione pool was depleted (Freeman & Mudd, 1981). This effect on the cellular glutathione level was also demonstrated in cultured lung cells (Rietjens *et al.*, 1985). Finally, DNA degradation was observed in strains of *Escherichia coli* (Hamelin & Chung, 1981) and *Saccharomyces cerevisiae* (Dubeau & Chung, 1984).

So far, no attempt has been made to correlate structural and functional consequences of ozone exposure of cells and the basis for the toxicity of ozone is still unknown. In order to elucidate the mechanism by which ozone causes severe cell damage and eventually cell death, we systematically studied the effect of exposure of cultured fibroblasts to ozone. In this report we

demonstrate that ozone exposure affects the activities of several key enzymes and the cellular level of both reduced and oxidized glutathione, suggesting that intracellular damage may play a role in ozone toxicity.

MATERIALS AND METHODS

NCTC mouse fibroblasts, clone L929, A.T.C.C. number CCL1, tissue culture products and newborn bovine serum was obtained from Flow Laboratories. All other chemicals were obtained from Boehringer, Sigma, Baker or Merck and were of analytical grade.

L929 mouse fibroblasts were grown as described before (Dubbelman & Van Steveninck, 1984). Ozone was generated by a Fisher Ozone Generator model 501. Ozone production was measured by titration with KI/Na₂S₂O₃/starch at pH 7.4. Prior to ozone exposure cells were washed twice with PBS and they were covered with 1 ml of the same buffer during exposure to ozone. The dishes were placed in a chamber, mounted on a Hetomix shaker. A flow of oxygen (0.4 ml/min) containing 25 μ mol of ozone/ml was maintained in the chamber. Ozone does not readily penetrate fluid overlying cultured cells and removal of the buffer dehydrates the cells. In the above mentioned system cells could be exposed to the ozone/oxygen mixture, without dehydration. Every 30 s the cells were exposed to buffer for 10 s.

After exposure the cell layer was washed twice with 150 mM-choline chloride/1 mM-Tris/HCl (pH 7.4) and once with 1 mM Tris/HCl (pH 7.4). The cells were scraped off into water. The K⁺ content of the cells was determined as described by Dubbelman & Van Steveninck (1984). The method of Pennington (1961) was used

Abbreviations used: AP, acid phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; PBS, iso-osmotic phosphate-buffered saline (pH 7.4); PK, pyruvate kinase; SDH, succinate dehydrogenase.

‡ To whom correspondence and reprint requests should be addressed.

for the assay of SDH activity and the method of Cooperstein & Lazarow (1951) for cytochrome *c* oxidase. PK was assayed as described by Hess & Wieker (1974). LDH activity was determined according to Bergmeyer & Berndt (1974). Measurement of GAPDH activity was performed by the method of Wu & Racker (1959). GDH was determined according to Strecker (1955). AP activity was measured with 4-methylumbelliferyl phosphate as substrate (1 mM) in a buffer containing 100 mM-sodium acetate and 2% Triton X-100 at pH 5.0. The reaction was stopped by adding a solution of 0.25 M-glycine/0.25 M- Na_2CO_3 (pH 10.5). The fluorescence of the liberated 4-methylumbelliferone was determined on an Aminco SPF-500 spectrophotofluorometer (excitation, 365 nm; emission, 450 nm). For the determination of β -glucuronidase activity the same method was used, but with naphthol-ASBI β -D-glucuronide as substrate (excitation, 405 nm; emission, 515 nm).

For the cytochemical assays of AP and β -glucuronidase the cells were grown on cover slips. The cell layer was washed with PBS after exposure to ozone, and fixed by treatment with 0.1% glutaraldehyde in PBS for 15 min at ambient room temperature. The fixed cells were washed twice with PBS and once with water. After drying the cells were permeabilized by incubation with acetone at -20°C for 15 min. After briefly washing with acetate buffer (pH 5.0), AP in the fixed cells was visualized according to the procedure of Barka & Anderson (1962), which uses hexazotized *p*-rosaniline as the capture agent of naphthol-ASBI liberated *in situ*. The incubation was for 1 h at room temperature. For β -glucuronidase, naphthol-ASBI glucuronide was used as the substrate and incubation was for 2 h at 37°C . The cells were washed with water, dried and mounted with Fluoromount for microscopic examination and photography.

The ATP content of the cells was measured with a Boehringer-Mannheim bioluminescence CLS kit in the presence of 0.5% Triton X-100. Protein was determined according to Lowry *et al.* (1951).

Determination of GSH and GSSG was performed by a modification of the method of Hissin & Hilf (1976). To 0.5 ml of cell suspension (10^6 cells) was added 0.2 ml of a solution containing 0.64% Triton X-100 and 1% HPO_3 . After centrifugation (10 min, 3000 rev./min) 0.25 ml of the supernatant was mixed with 0.25 ml of 0.1 M-sodium phosphate buffer containing 5 mM-EDTA (pH 8.0), and 300 μl of the mixture was added to 1.6 ml of the phosphate/EDTA buffer (pH 8.0) and 100 μl of *o*-phthalaldehyde (0.1% in methanol). After 15 min at room temperature the fluorescent GSH adduct was determined (excitation, 350 nm; emission, 420 nm). For the determination of GSSG, 0.25 ml of the supernatant was added to 0.1 ml of 0.04 M-*N*-ethylmaleimide in 0.1 M-NaOH. After 30 min incubation at room temperature, 150 μl of 0.1 M-NaOH was added, and 300 μl of this mixture was incubated with 1.6 ml of phosphate/EDTA buffer and 100 μl of *o*-phthalaldehyde at room temperature for 15 min and the fluorescence was measured (excitation, 350 nm; emission, 420 nm).

Leakage of soluble proteins to the medium was monitored by protein measurements in the medium. In control experiments it appeared that exposure of proteins to ozone under the present experimental conditions did not interfere with protein determination according to Lowry *et al.* (1951). Leakage of soluble

proteins was related to the amount of protein liberated into the supernatant after scraping of the cells in water and sonication.

In some experiments enzyme inactivation was studied after a preceding destruction of the cell structure. For that purpose cells were scraped in 1 ml of PBS and disrupted by sonication for 15 s at 0°C with a Branson sonifier (75 W). After sonication the suspension was exposed to ozone as described above for intact cells.

For all ATP, GSH and enzyme activity measurements, values were within the range $\pm 8\%$, with two to five independent experiments for each datum point.

RESULTS

During the experiments described below, the cells were exposed to a mixture of oxygen and ozone. In control experiments cells were exposed to pure oxygen only for periods up to 2 h. It appeared that this treatment had no influence on any of the parameters studied in the present investigations.

Upon exposure of murine L929 fibroblasts to the mixture of oxygen and ozone the cells immediately started to leak K^+ . When ozone exposure was discontinued, the velocity of K^+ leakage decreased sharply, but was still significant (Fig. 1). Ozone exposure also caused a relatively minor leakage of soluble proteins into the medium (Fig. 1).

The effect of ozone on the activity of three cytosolic enzymes is shown in Fig. 2. In control experiments it appeared that under the incubation conditions, the enzyme reactions were linear both with the amount of cell suspension and with time. GAPDH and PK activity decreased rather fast upon exposure to ozone, GAPDH being the most sensitive. When 60% of the K^+ had leaked

Fig. 1. K^+ and protein leakage with L929 fibroblasts as a function of ozone exposure time

●, K^+ leakage during continuous ozone exposure; ○, K^+ leakage, ozone discontinued after 15 min; ▲, protein leakage. Initial cellular K^+ content, $0.97 \mu\text{mol}/\text{mg}$ of protein. Each point represents the mean of three to five experiments.

Fig. 2. Activity of cytosolic enzymes upon exposure of L929 fibroblasts to ozone

○, GAPDH; △, PK; ■, LDH. Initial enzyme activities: GAPDH, $0.27 \mu\text{mol/min}$ per mg of protein; PK, $0.34 \mu\text{mol/min}$ per mg of protein; LDH, $1.0 \mu\text{mol/min}$ per mg of protein. Each point represents the mean of three to five experiments.

out of the cells GAPDH was almost completely inhibited and the PK activity was decreased to 35% of its initial value. Only a small effect upon the LDH activity was observed: a 20% decrease in activity when 60% of the K^+ had leaked out of the cells. No LDH, GAPDH or PK activity outside the cells could be detected.

Two lysosomal enzymes were studied, the membrane-bound AP and the intralysosomal β -glucuronidase. The activity of both enzymes started to decline when about 60% of the K^+ had leaked out of the cells (Fig. 3). The

Fig. 3. Activity of lysosomal enzymes upon exposure of L929 fibroblasts to ozone

○, AP; ●, β -glucuronidase. Initial enzyme activities: AP, 52 nmol/min per mg of protein; β -glucuronidase, 21 nmol/min per mg of protein. Each point represents the mean of two experiments.

Fig. 4. Activity of mitochondrial enzymes upon exposure of L929 fibroblasts to ozone

○, SDH; ●, GDH; ▲, cytochrome *c* oxidase. Initial enzyme activities: SDH, 13 nmol/min per mg of protein; GDH, 44 nmol/min per mg of protein; cytochrome *c* oxidase, 70 nmol/min per mg of protein. Each point represents the mean of two to five experiments.

localization of these two enzymes was studied with cytochemical methods. The lysosomal staining patterns of both AP and β -glucuronidase did not change upon ozone exposure.

In further experiments the inactivation by ozone of the mitochondrial enzymes cytochrome *c* oxidase, SDH and GDH was followed. SDH activity slightly decreased, whereas no effect on the GDH activity was observed. The cytochrome *c* oxidase activity only started to decline after 60% of the K^+ had leaked out of the cells (Fig. 4).

Fig. 5. Enzyme activities in a sonicated cell suspension during exposure to ozone

○—○, GAPDH; △, PK; ■, LDH; ●, GDH and SDH; ▲, AP; □, β -glucuronidase; ○—○, cytochrome *c* oxidase. Each point represents the mean of three experiments.

In an attempt to establish the sensitivity of the enzymes to ozone *per se*, the decrease in enzyme activity in intact cells was compared with the decrease in activity in a suspension of sonified cells (Fig. 5). In suspension GAPDH and PK were also the most susceptible to ozone and GAPDH activity was almost completely inhibited in 2 min. The other enzymes were inactivated as well, except for cytochrome *c* oxidase. The activity of this enzyme decreased to 40% and then an increase in activity was observed. In order to see whether this unexpected behaviour was an intrinsic property of this enzyme, the effect of ozone on a preparation of purified enzyme dissolved in 50 mM-sodium phosphate (pH 7.4) was studied. In these experiments no such effect was found: enzyme activity decreased progressively during ozone exposure.

The cellular level of GSH was decreased after exposure of the cells to ozone. An increase in the GSSG level was observed, followed by a decrease (Fig. 6). Thiol compounds are very susceptible to oxidation by ozone. GSH and GSSG, when exposed in solution, both reacted instantaneously with ozone. GSH and GSSG could not be detected outside the cells. The initial GSH content amounted to 50 nmol/mg of protein. The accuracy of the method to determine GSSG in biological samples is still under discussion (Rietjens *et al.*, 1985), and therefore the absolute cellular amount of GSSG was not quantified.

The ATP content of the cells amounted to 3.2 nmol/mg of protein and did not decrease upon exposure to ozone.

In a final series of experiments cells were exposed to ozone during 20 or 50 min and subsequently incubated in medium for 3 h. It appeared that during this post-incubation residual enzyme activities remained unchanged. Apparently enzyme damage was non-progressive after discontinuation of ozone exposure, but also irreversible. After prolonged post-incubation up to 24 h, a slight increase of enzyme activity was observed, but concomitantly the number of cells increased.

Fig. 6. Effect of ozone exposure on the glutathione levels of L929 fibroblasts

○, GSH; ●, GSSG. Initial GSH concentration: 50 nmol/mg of protein. Each point represents the mean of three experiments.

DISCUSSION

A variety of biochemical and histochemical techniques have been used in a systematic investigation of the toxic effects of ozone on L929 fibroblasts in culture. The ultimate aim is to elucidate the mechanism by which ozone causes cell death.

K⁺ leakage is a very sensitive parameter of ozone-induced damage (Fig. 1); therefore K⁺ leakage will be used as an 'internal standard' of membrane damage throughout these studies. An increase in cell membrane permeability for K⁺ is often the first phenomenon that can be observed when cells are exposed to various oxidative agents (Van der Zee *et al.*, 1985). Other investigators have used leakage of a cytosolic enzyme, like LDH, as a marker to detect cell membrane damage (Christensen *et al.*, 1983). This is, however, an unsuitable parameter to monitor ozone-induced membrane damage. Firstly, after 50 min of exposure to ozone, the intracellular LDH activity was decreased by only 20%, whereas 60% of the intracellular K⁺ had already leaked out of the cells (Figs. 1 and 2). This indicates that K⁺ leakage is *a priori* a much more sensitive parameter than LDH leakage to measure membrane damage. Secondly, after 50 min of exposure to ozone, no LDH activity could be detected in the medium. But even if LDH activity would have been found outside the cells, LDH leakage could not have been quantified (e.g. as a percentage of initial intracellular activity) due to the oxidative inactivation of the enzyme intracellularly and, even faster (Fig. 5), after leakage to the medium.

When intact cells were exposed to ozone, the activity of GAPDH and PK was strongly inhibited (Fig. 2). The inactivation of GAPDH was faster than the increase in K⁺ leakage. This indicates that when L929 cells are exposed to ozone, GAPDH, which is located in the cytoplasm or bound to the cell membrane, is more sensitive than the membrane site responsible for the increased K⁺ permeability. Inactivation of PK proceeded at the same rate as the K⁺ leakage. The inhibition of LDH was rather slow compared with that of GAPDH and PK (Fig. 2).

A possible mechanism of inactivation of these cytoplasmic enzymes would be leakage to the medium, with subsequent rapid inactivation. However, leakage of soluble proteins as a consequence of exposure to ozone is a relatively slow process (Fig. 1), leading to a leakage of about 10% after an exposure period of 50 min, much lower than the percentage of inactivation of, e.g., GAPDH (85%). Considering the *M_r* values of LDH, GAPDH and PK (140 000, 144 700 and 237 000 respectively), a preferential leakage of these enzymes as compared with the other cytoplasmic proteins seems unlikely. Therefore it seems highly probable that most of the enzyme inactivation actually occurs within the cells.

The activity of cytochrome *c* oxidase, AP and β -glucuronidase did not decrease until 60–70% of the K⁺ had leaked out (Figs. 3 and 4). At this point GAPDH and PK were completely inactivated and LDH was inhibited by 30%. SDH was slightly more sensitive than the other mitochondrial and both lysosomal enzymes. Apparently, enzymes localized in lysosomes and mitochondria were affected by ozone only after a prolonged exposure time, when already severe damage had been induced to the cells and an overall deterioration of the cell started to occur. This is in accordance with our finding that the

histochemical staining patterns of both lysosomal enzymes did not change. Release of lysosomal enzymes has been implicated in cell death induced by oxidative stress (Allison *et al.*, 1966; Santus *et al.*, 1983). It is obvious that this mechanism does not play a role in ozone-induced deterioration of L929 cells.

The observed sequence of enzyme inactivation indicates that localization of the enzymes is an important factor to consider. This conclusion is strengthened by the experiments with the sonified cell suspension. It appeared that all enzymes are basically susceptible to ozone (Fig. 5), but again GAPDH was by far the most sensitive enzyme.

The susceptibility of proteins to oxidative damage will depend to a large extent on the availability of sensitive residues and on the role of these residues in the function of the protein. For example, the inactivation of GAPDH by ozone could be correlated with oxidation of the active site -SH residue (Knight & Mudd, 1984). Oxidation of other -SH groups and tryptophan, methionine and histidine occurred at the same time, but loss of activity depended solely on active site oxidation. An effect of ozone on the active site of lysozyme was observed by Kuroda *et al.* (1975). One of the six tryptophan residues at the active site was oxidized, with concomitant loss of enzyme activity. It is apparent that oxidation of amino acids at the active site plays a significant role in enzyme inactivation by ozone. The importance of accessibility of sensitive residues is illustrated by the decline in LDH activity. LDH contains 13 thiol groups, three of which are required for enzymic activity (Grüber *et al.*, 1962). However, LDH is not very sensitive to ozone (see, e.g., Fig. 5), indicating that the essential -SH groups of the enzyme are not readily accessible to ozone.

A peculiar phenomenon was observed in the case of cytochrome *c* oxidase activity in the sonicated cell suspension (Fig. 5). After a decrease in activity to 40%, an increase was observed. This phenomenon was not found in experiments with the purified enzyme. In intact cells cytochrome *c* oxidase is bound to the inner mitochondrial membrane. It may be possible that in the sonicated cell suspension the enzyme is still bound to fragments of this membrane and that modification of membrane constituents accounts for the observed effect. A change in the attached membrane may also induce a change in the conformation of the protein and subsequently in the enzyme activity. In most cases protein as well as lipid oxidation will cause inhibition of the enzyme, but for cytochrome *c* oxidase they may counteract each other and bring about a reactivation of the enzyme.

Other factors besides enzyme inactivation that may be of importance in the deterioration of the cells and ultimately in cell death are depletion of intracellular compounds like GSH and ATP. Although ATP itself is rapidly degraded by ozone (Shinriki *et al.*, 1984), the intracellular pool of ATP proved to be rather insensitive to ozone exposure. This indicates that energy depletion of the cells did not occur and thus cannot be responsible for ozone-induced cell death.

Much attention has been focused on the effects of oxidative stress on the glutathione level in cells, since this may play an important role in the defence mechanism of cells against oxidative species. In solution GSH and GSSG were both readily oxidized by ozone. The same results were obtained by Menzel (1971) and Mudd *et al.*

(1969). In intact cells the GSH concentration declined, but the GSSH level first increased (Fig. 6), indicating that GSH was converted to GSSH. This may be due to direct oxidation or by reduction of other cellular components by GSH, yielding GSSG. Longer exposure to ozone resulted in a decline of both the GSH and the GSSG concentration, without a concomitant increase of GSH or GSSG in the buffer covering the cells. Thus this indicates either conversion of GSH or GSSG to further oxidation products, not reactive with the GSH and GSSG reagent(s), or the formation of protein-GSH mixed disulphides. The latter mechanism was demonstrated, e.g., by Collison *et al.* (1986) in heart cells exposed to oxidative challenge by *t*-butylhydroperoxide or diamide. Because GSH and GSSG might both be readily oxidized after leakage into the medium, it cannot be ruled out that leakage is partly responsible for the decrease of GSH and GSSG.

This study emphasizes the deleterious effect of ozone on proteins and especially on thiol groups, not only in its effect on the glutathione level in the cells but also in its role in the inactivation of several enzymes. Our results imply that both K⁺ leakage and inactivation of certain cytoplasmic enzymes may be involved in ozone-induced cell death. Further experiments to elucidate the mechanism of ozone-induced cell death are necessary.

We are much indebted to Cock Smit and Lalita Ramdin-Adhin for assistance with the experiments. This work was supported by a grant of the Netherlands Organization for the Advancement of Pure Scientific Research, Z.W.O. (grant number 98-61).

REFERENCES

- Allison, A. C., Magnus, I. A. & Young, M. R. (1966) *Nature* (London) **200**, 874-878
- Barka, T. & Anderson, P. J. (1962) *J. Histochem. Cytochem* **10**, 741-753
- Bergmeyer, H. U. & Berndt, E. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 778-780, Academic Press, New York
- Christensen, T., Sandquist, T., Feren, K., Waksvik, H. & Moan, J. (1983) *Br. J. Cancer* **48**, 35-43
- Collison, M. W., Beidler, D., Grimm, L. M. & Thomas, J. A. (1986) *Biochim. Biophys. Acta* **885**, 58-67
- Cooperstein, S. J. & Lazarow, A. (1951) *J. Biol. Chem.* **189**, 665-675
- Dubbelman, T. M. A. R. & Van Steveninck, J. (1984) *Biochim. Biophys. Acta* **771**, 201-207
- Dubeau, H. & Chung, Y. S. (1984) *Mol. Gen. Genet.* **197**, 120-124
- Freeman, B. A. & Mudd, J. B. (1981) *Arch. Biochem. Biophys.* **208**, 212-220
- Grüber, W., Warzecha, K., Pfeleiderer, G. & Wieland, Th. (1962) *Biochem. Z.* **336**, 107
- Hamelin, C. & Chung, Y. S. (1981) *Mol. Gen. Genet.* **184**, 560-561
- Hess, B. & Wicker, H. J. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 509-510, Academic Press, New York
- Hissin, P. J. & Hilf, R. (1976) *Anal. Biochem.* **74**, 214-226
- Knight, K. L. & Mudd, J. B. (1984) *Arch. Biochem. Biophys.* **229**, 259-269
- Kuroda, H., Sakiyama, F. & Nasita, K. (1975) *J. Biochem. (Tokyo)* **78**, 641-651
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. I. (1951) *J. Biol. Chem.* **193**, 265-275

- Menzel, D. B. (1971) *Arch. Environ. Health* **23**, 149–153
- Menzel, D. B. (1976) in *Free Radicals in Biology* (Pryor, W. A., ed.), vol. 2, pp. 181–201, Academic Press, New York
- Menzel, D. B. (1984) *J. Toxicol. Environ. Health* **14**, 183–204
- Morgan, D. L. & Wenzel, D. G. (1985) *Toxicology* **36**, 243–251
- Mudd, J. B., Leavitt, R., Ongun, A. & McManus, T. T. (1969) *Atmos. Environ.* **3**, 669–682
- Pauls, K. P. & Thompson, J. E. (1981) *Physiol. Plant.* **53**, 255–262
- Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654
- Rietjens, I. M. C. M., Alink, G. M. & Vos, M. E. (1985) *Toxicology* **35**, 207–217
- Santus, R., Kohen, C., Kohen, E., Reijftmann, J. P., Morliere, P., Dubertret, L. & Tocci, P. M. (1983) *Photochem. Photobiol.* **38**, 71–77
- Shinriki, N., Ishizaki, K., Sato, S., Miura, K., Sawadaishi, K. & Ueda, T. (1984) *Chem. Pharm. Bull.* **32**, 3636–3640
- Strecker, A. J. (1955) *Methods Enzymol.* **2**, 220
- Van der Zee, J., Dubbelman, T. M. A. R. & Van Steveninck, J. (1985) *Biochim. Biophys. Acta* **818**, 38–44
- Wu, R. & Racker, E. (1959) *J. Biol. Chem.* **234**, 1029–1035

Received 28 July 1986/13 October 1986; accepted 14 November 1986