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THE INHIBITION OF CHROMATIN SOLUBILIZATION BY PROTEOLYTIC INHIBITORS

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Summary: Several inhibitors of the proteolysis of histone H1, were examined for their effect on chromatin solubilization in a rabbit thymus nuclear incubation. Tosyl-lysine chloromethyl ketone and Tosylamino-phenylethyl sulfonyl fluoride were inhibitory while leupeptin was without effect.

The realization that the Rec A protein of E. coli carries out two apparently unrelated reactions, a proteolysis and an ATP hydrolysis (1), has prompted us to report some recent results with a mammalian system which on the surface appear to have a parallel. We wish to report on the inhibitory activity of two protease inhibitors, TLCK* and TPCK, in a system which involves the formation of a soluble chromatin complex from insoluble chromatin fibers. The soluble chromatin is produced by incubation of a rabbit thymus nuclear lysate or washed chromatin fibers in a Tris-sulfate buffer with 0.001M Mg^{++}. Some characteristics of the reaction and of the chromatin product have been presented previously [2-5]. In an endeavor to inhibit the slight proteolysis of histone H1 which occurs in this reaction we resorted to the use of several common proteolytic inhibitors. We have reported [6] that PMSF was ineffective while leupeptin and TLCK were active in blocking the breakdown of histone H1 in the chromatin incubation. Neither of these inhibitors appeared to effect the solubilization of the chromatin. The formation of the soluble chromatin from insoluble chromatin fibers presumably is the result of a nucleolytic activity. The basis for this supposition is the observation that as the reaction progresses the size of the DNA in the soluble complex is reduced [2,3,4,7]. We have had the occasion to examine more closely the effect of TLCK and TPCK on the chromatin

*abbreviations: TLCK - tosyl - lysine chloromethyl ketone
TPCK - tosylamino - phenylethyl chloromethyl ketone
PMSF - phenylmethyl sulfonyl fluoride

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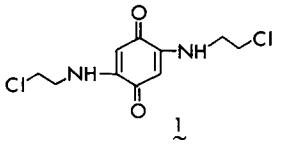
the formation of aziridinium salt 3 in the cellular system and coincides well with the above.



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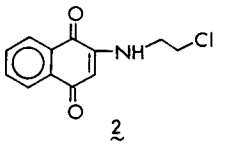

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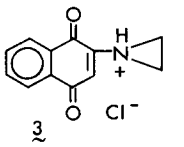

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We confronted the question of why compound 1 or 2 is bioactive and report here on the enzyme-induced aziridine formation from 2-chloroethylaminobenzoquinones or naphthoquinones.

MATERIALS AND METHODS

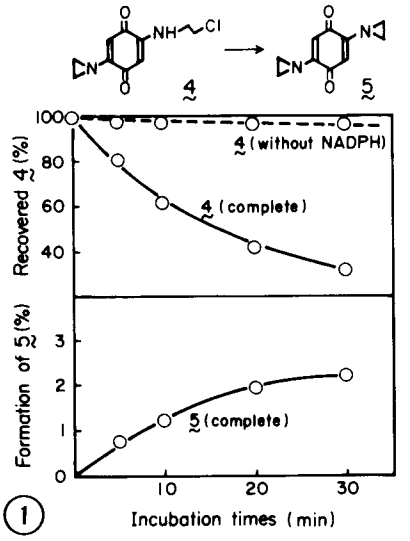
2,5-Bis(2-chloroethylamino)-p-benzoquinone (1), 2,5-bis(1-aziridinyl)-p-benzoquinone (5), 2-chloroethylaminonaphthoquinone (2), and 1-aziridinylnaphthoquinone (6) were prepared by the method of Petersen et al. (1). 2-(2-Chloroethylamino)-5-aziridinyl-p-benzoquinone (4) and 2-bromoethylaminonaphthoquinone (7) were prepared from the corresponding aziridines 5 and 6 with concentrated HX acid in acetonitrile. 4, m.p. 115-116°C, orange plates ($CHCl_3$); 7, m.p. 182-3°C, red plates (EtOH). 4-Chlorobutylaminonaphthoquinone (8) was prepared by addition reaction of 4-aminobutanol to naphthoquinone followed by chlorination with thionylchloride in dichloroethane. m.p. 139-140°C, red plates (MeOH). Pyrrolidinylnaphthoquinone (9) was prepared by addition reaction of pyrrolidine on naphthoquinone in ethanol (1). m.p. 158.5-159.5°C, red plates (MeOH). Authentic sample of 12 (n = 4) was prepared by treating 9 with $NaBH_4$ followed by the dimethylsulfate reaction. Colorless needles, m.p. 85-86°C for 12 (n = 4) (MeOH).

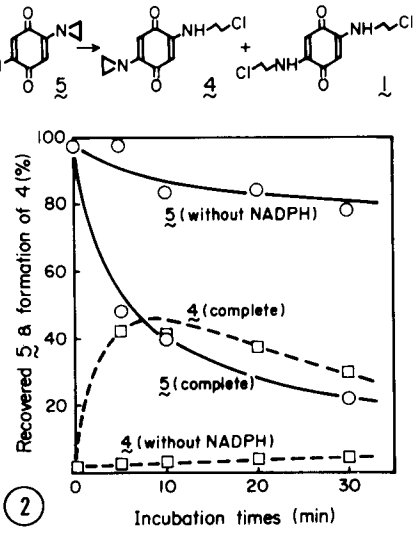
General procedure of transformation of chloroethylamino- or aziridinyl-p-benzoquinones or naphthoquinones in microsomal suspension. Washed liver microsomes were prepared from normal SLC-Wistar strain male rats, weighing about 300 g, by the differential centrifugation method (3). The protein concentration of the microsomes was determined by the biuret reaction (3) using bovine serum albumin as a standard.

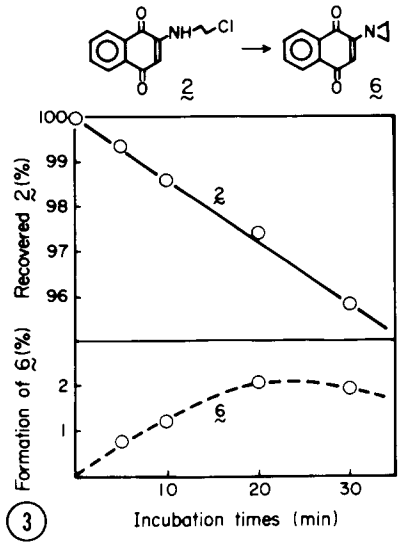
The metabolic reactions were carried out in a complete system consisting of 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl, 10 mM $MgCl_2$, 1 mM nicotinamide, 1 mM pyrophosphate, 30 mg of protein of microsomes, 0.0026 mM of substrates and an NADPH-generating system (0.9 mM NADP, 10 mM glucose-6-phosphate and 12.5 units of glucose-6-phosphate dehydrogenase) in a final volume of 5 ml. Unless otherwise stated, incubation was carried out aerobically at 37°C with moderate shaking. This procedure was almost the same as that described previously in the study of the aziridine derivative (4). To stop the enzymatic reaction, aliquots portion of the reaction mixture were added to twice the volume of ice-cooled acetonitrile. The top clear solution obtained by centrifugation was analyzed by HPLC using Nucleosil$_{10}$ C_{18} column. The reaction products were identified mainly by GC/MS or comparison of the physical data with those of authentic samples. The yields of the reaction products were calculated from the HPLC peak areas.

RESULTS

A) Reaction of 2-(2-chloroethylamino)-5-aziridinyl-p-benzoquinone 4 and 2,5-bis(1-aziridinyl)-p-benzoquinone 5 in the complete, NADPH-free or







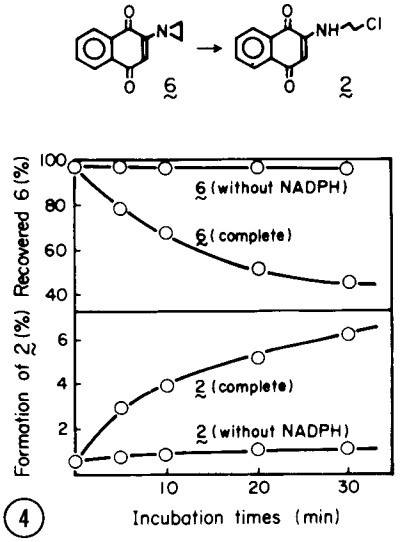


Fig. 1. Reaction of 4 in the complete or NADPH free system (37°C)

Fig. 2. Reaction of 1 in the complete or NADPH free system (37°C)

Fig. 3. Reaction of 2 in the complete system (37°C)

Fig. 4. Reaction of 6 in the complete or NADPH free system (37°C)

microsomes free system (Figs. 1 and 2). At first, 4 was treated with rat liver microsomes in the complete system. The reaction products were 2,5-bis(1-aziridinyl)-p-benzoquinone 5 and a small amount of 2,5-bis(2-chloroethylamino)-p-benzoquinone 1. The amounts of recovered 4 and the yield of 5 from the reaction mixture incubated for appropriate periods are shown in Fig. 1. The NADPH-free or microsome-free system showed no reaction on 4 nor

solubilization. This report concerns the inhibitory action of these two compounds on this reaction.

Methods

Rabbit thymus nuclei were isolated from frozen tissue (Pel-Freeze, Rogers, Arkansas) as described [2,4]. The nuclei were suspended in sucrose at a rate of 10 volumes per 1 volume of packed nuclei. The suspension was maintained at 20-22° C for 20 min, and then 2.0 ml was mixed with 2.08 ml of 0.08 M K_2SO_4-0.04 M Tris-sulfate pH 7.6 and 0.083 ml 0.05 M $MgSO_4$ (final Mg^{++} = 0.001 M). After 2 mins the supernatant solution was decanted from the gel-like aggregate and discarded. The aggregate was washed with 12 ml of 0.04 M K_2SO_4-0.02 M Tris-sulfate - 0.001 M Mg^{++} and after decantation mixed with 3.6 ml of this buffer. All the foregoing operations were at 20-22° C. When specified, inhibitor was included in the original lysis, the wash and the incubation. After incubation of the aggregate for 30 mins at 37° C, 0.1 M EDTA was added (final EDTA = .005 M), the mixture cooled to 0° C and centrifuged at 3000 rpm for 30 mins. The supernatant was removed, an aliquot diluted with buffer and the absorbance read at 259 nm. The leupeptin was a gift from T. Sugimura, National Cancer Institute, Tokyo, Japan. Other reagents were commerical products.

Results and Discussion

The results of a typical experiment are shown in Fig. 1. The absorbance reading with no inhibitor added corresponds to about 60% solubilization of the chromatin. It can be seen that leupeptin is without effect as previously reported [6] while TLCK and TPCK are both effective inhibitors. Other so-called proteolytic inhibitors were also examined. Soybean trypsin inhibitor (0.2 mg/ml) and pepstatin (0.05 mg/ml) were not inhibitory whereas, with the following, some inhibition was observed: PMSF, $1x10^{-3}$M, 45%: p-hydroxymercuribenzoate, $1x10^{-3}$M, 50% and $NaHSO_3$, $1x10^{-2}$M, 30%. It should be emphasized that none of the aforementioned inhibitors were effective in preventing the

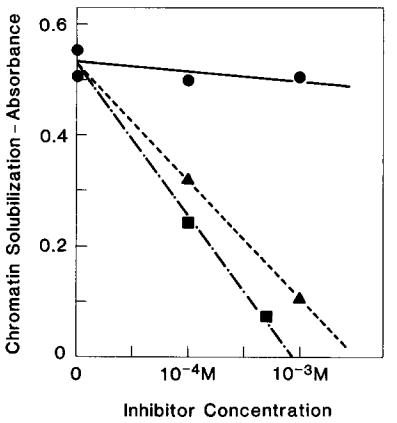


Fig. 1. The inhibition of chromatin solubilization by TLCK and TPCK. Incubation was at 37° C for 30 minutes. Ordinate: absorbance of a 0.1→5.0 dilution of the supernatant solution from the nuclear lysate incubation. Readings have been corrected for zero time values. ●-●, Leupeptin: ▲-▲, TLCK: ■-■, TPCK.