The Effect of Acrolein on L-Asparaginase 2 from *Escherichia coli* (35344)

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L-Asparaginase 2 has attracted some attention as an antitumor agent (1–3) and its reaction to cigarette smoke was considered of interest.

Inhibition studies on asparaginase from *E. coli* were carried out using cigarette smoke and a number of reactive smoke components, including formaldehyde, acetaldehyde, glyoxal, acrolein, hydroquinone, 1,4-benzoquinone, nicotine, phenol, and *o*-cresol. Of these only acrolein, in concentrations 50 times greater than that found in the smoke of a typical cigarette, caused any inhibition. Due to the conflicting evidence for the requirement of free thiol groups for activity in asparaginases, the effect on the enzyme of acrolein and two other –SH specific reagents, *p*-hydroxymercuribenzoate and sodium arsenite, were studied in some detail.

Initial experiments were performed on a pure, commercial sample of the enzyme which contained asparaginases 1 and 2. In this form, however, the enzyme was found to be extremely labile and had to be stabilized by addition of bovine serum albumin prior to assay. Due to this instability of the purified preparation and also since our interest was in asparaginase 2, the inhibition studies were carried out on a partially purified preparation from *E. coli* which contained only 3% of asparaginase 1.

Materials and Methods. The culture of *E. coli* was grown with shaking on a rotary shaker (100 rpm) in a medium containing peptone, 1.0%; beef extract, 0.6%; KH_2PO_4, 0.33% (4). A 2% Hycase–0.02% yeast extract medium (5) was also used. The cells were harvested in a refrigerated centrifuge, lyophilized, and the enzyme extracted by sonication of a heavy suspension of lyophilized powder. Partial purification of the enzyme up to the $(NH_4)_2SO_4$ stage was carried out as descibed by Mashburn and Wriston (6). Most of the L-asparaginase 2 activity was present in the 100% saturation $(NH_4)_2SO_4$ precipitate, which was dissolved in distilled water and initially dialyzed against repeated changes of deionized water until salt-free, and finally against physiological saline. This preparation (sp act 1.38 IU/mg of protein) represented a 5-fold purification over the sonicate, and contained only about 3% L-asparaginase 1.

Determination of protein was carried out by the spectrophotometric method of Groves *et al.* (7).

The two forms of L-asparaginase were determined by the procedure and equations given by Campbell *et al.* (8). The unit of enzyme activity was been defined as that amount of enzyme which will release 1 μmole of ammonia in 1 min at the initial maximum rate.

In inhibition studies the enzyme and substance under study were preincubated for 20 min, after which, appropriate dilution was carried out and the enzyme assays were performed at pH 8.4 and 5.0. In the case of *p*-hydroxymercuribenzoate, diluted enzyme was preincubated with the reagent, since the latter, even in high concentration, does not interfere with the determination of ammonia. Acrolein inhibition was studied by preincubating the enzyme with acrolein at pH values of 8.4 and 5.0 for varying lengths of time prior to assay. Preincubation mixtures contained 28 IU of L-asparaginase 2; 50 μmoles of acrolein; and 25 m*M* borate or acetate buffers, pH 8.4 and 5.0, respectively. At the end of the appropriate period, the mixture was diluted 1:200 using physiological saline and assayed for L-asparaginase activity in a

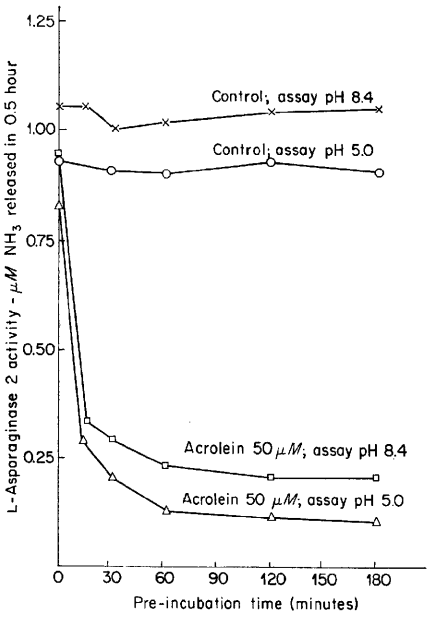

FIG. 1. Inhibition of L-asparaginase 2 by acrolein (preincubation at pH 8.4).

reaction mixture containing 0.2 m*M* borate or acetate buffer, pH 8.4 and 5.0, respectively, and 20 μmoles L-asparagine (4).

Results and Discussion. When commercially available L-asparaginase (containing both L-asparaginases 1 and 2) is preincubated for 20 min with acrolein at a concentration of 5 μmoles, no inhibition was seen, but when the acrolein concentration was raised to 50 μmoles in the preincubation mixture about 40% inhibition was observed. Partially purified L-asparaginase 2 obtained in this laboratory gave similar results. No inhibition of the enzyme was found when it was preincubated for 20 min with 5 μmoles of acrolein, but when the acrolein concentration was raised to 50 μmoles in the preincubation mixture, about 30% inhibition was observed. That the inhibition observed with acrolein was not due to hydroquinone which is added to it as stabilizer was also confirmed. Hydroquinone at a concentration of 2.5 μmoles in the preincubation mixture (100 times greater than that normally added to acrolein) was tested and found not to inhibit the enzyme.

The effect of pH of the preincubation mixture on inhibition was studied and revealed that preincubation of the enzyme and acrolein at pH 8.4 resulted in a more marked inhibition than preincubation at pH 5.0. This is clearly illustrated in Figs. 1 and 2. Greater inhibition at pH 8.4 indicates that ionization of the –SH group, which would be greater at pH 8.4 than at pH 5.0 (p*K* of the –SH group is between 8 and 9), plays an important role in reaction with acrolein. Under basic conditions –SH groups have been shown to react with the double bond of acrolein according to the following equation (9): $RSH + CH_2 = CH\text{–}CHO \rightarrow RSCH_2\text{–}CH_2CHO$.

Since inhibition by acrolein indicated a requirement for free thiol groups for enzyme activity, other thiol reagents such as sodium arsenite and p-hydroxymercuribenzoate were also examined for their ability to inhibit L-asparaginase 2. Neither caused inhibition as is shown in Table 1.

It is difficult to explain why one –SH reagent, acrolein, inhibits this enzyme while others like *p*-hydroxymercuribenzoate and sodium arsenite do not. Jayaram *et al.* (10) have obtained similar conflicting results in their studies with L-asparaginases from two strains of *Mycobacterium tuberculosis*. They observed that while sodium arsenite and cadmium ions inhibited the "pH 9.6 enzyme"

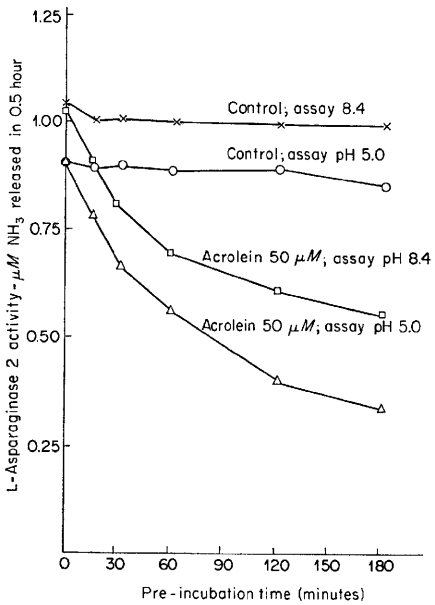

FIG. 2. Inhibition of L-asparaginase 2 by acrolein (preincubation at pH 5.0).

TABLE I. Effect of Sulfydryl Reagents on the Activity of L-Asparaginase 2.

Reagent	Conc of reagent in preincubation mixture (μmoles/ml)	Component (μmoles /mg of protein in preincubation mixture)	Inhibition (%)
Acrolein	50	9.4	30
Sodium arsenite	50	9.4	Nil
p-Hydroxymercuribenzoate	3.34	95	Nil

present in these strains, *p*-chloromercuribenzoate did not; *p*-chloromercuribenzoate inhibited the "pH 9.0" enzyme in these strains, while arsenite did not. Recently, Cedar and Schwartz (11) also showed that *p*-hydroxymercuribenzoate, iodoacetate, iodoacetamide and *N*-ethylmaleimide failed to inhibit L-asparaginase 2 from *E. coli*.

It is pertinent to refer to other inhibition studies done with L-asparaginases. Tower *et al.* (12), using purified guinea pig serum enzyme, found that L-glutamic acid, L-glutamine, L-aspartic acid, NH_4Cl, α-alanine, oxaloacetate, α-ketoglutarate, pyruvate, Mg^{2+}, Ca^{2+}, Mn^{2+}, fluoride, cyanide, *N*-ethylmaleimide, and pyridoxal PO_4 did not inhibit this enzyme, while *p*-chloromercurisulfonate, $HgCl_2$, Zn^{2+}, and polyvinylpyrrolidone did. Grossowicz and Halpern (13) observed that D-asparagine inhibits competitively the L-asparaginase obtained from *Mycobacterium phlei*. Similarly, Ott (14) studied the effect of various inhibitors, which included *p*-chloromercuribenzoate and other sulfydryl reagents, on L-asparaginases from *Mycobacterium smegmatis* and *M. tuberculosis* $H_{37}R_a$ and found that, with the exception of D-asparagine, none inhibited these enzymes. On the other hand, Manning and Campbell (15) found that L-asparaginases obtained from *Bacillus coagulans* and *Bacillus stearothermophilus* were inhibited by *N*-ethylmaleimide and *p*-chloromercuribenzoate indicating that the enzymes require sulfydryl groups for their activity. From the above, it may be conjectured that different organisms produce a variety of L-asparaginases and their recognition and separation is of considerable interest.

Summary. Inhibition studies, carried out using a partially purified L-asparaginase 2 preparation, have indicated that tobacco smoke and a number of reactive smoke components do not cause inhibition. Of the smoke components studied, only acrolein was inhibitory and this inhibition was found to be more marked at pH 8.4 than at pH 5.0. The –SH specific reagents, sodium arsenite and *p*-hydroxymercuribenzoate did not inhibit the enzyme.

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