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GLUTAMINE SYNTHETASE
VI. MECHANISM OF THE DITHIOL-DEPENDENT INHIBITION BY ARSENITE

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SUMMARY

Dithiol-dependent inhibition by arsenite has been demonstrated in glutamine synthetase (L-glutamate:ammonia ligase (ADP), EC 6.3.1.2) obtained from various sources: from Neurospora to mammalian tissues. With a partially purified enzyme preparation from rat liver, α,ω-dimercaptoalkanes, heterocyclic and aromatic dimercapto compounds have been compared for their ability to activate the enzyme and to mediate arsenite inhibition. BAL and 1,2-dimercaptoethane proved to be the most effective dithiols in both respects.

Equimolar quantities of BAL and arsenite produced the greatest inhibition of glutamine synthetase. Excess arsenite did not cause an additional inhibition, but excess BAL diminished it. Cysteine, though less effective than BAL, also reversed the inhibition.

Mapharside inhibited the enzyme at low concentrations and required no dithiol. Inhibition by either mapharside or *p*-arsenosobenzoate could be prevented more effectively by BAL than by cysteine. Inhibition by the organic arsenicals increased progressively with the length of time of prior incubation with the enzyme; that by arsenite, however, showed little increase. In contrast, prior incubation with *o*-iodosobenzoate produced a spontaneous reactivation of the enzyme with time.

Both Cd^{2+} and mercurial treatments of glutamine synthetase altered the course of arsenite inhibition. Cd^{2+} treatment appeared to interfere with arsenite inhibition; mercurial treatment enabled cysteine, as well as BAL to mediate the inhibition.

Evidence has been presented to show that the enzyme–arsenite complex dissociates readily. Moreover, kinetic analysis gives a value for K_i as high as K_m for hydroxylamine and adenosinetriphosphate. These results have invalidated the currently held assumption that dithiol-dependent arsenite inhibition results in the formation of a relatively stable ring structure with two closely juxtaposed sulfhydryl groups of the enzyme molecule. Instead, a mechanism has been proposed for the dithiol-mediated inhibition by arsenite, which entails reaction with only one sulfhydryl group of glutamine synthetase.

INTRODUCTION

Following the discovery of BAL as an antidote for arsenical vesicants, THOMPSON *et al.*[1–4] pioneered a series of systematic investigations on the effect of arsenicals on brain pyruvate oxidase, and the prevention as well as reversal of the inhibition by a number of dithiols. These investigations led to the demonstration that the arsenic of lewisite combined with the sulfur of kerateine in the ratio of 1 As to 2 S (see ref. 2), giving rise to the hypothesis that the arsenical acts by reacting with two closely placed mercapto groups in the protein to form a relatively stable ring structure. This "dithiol theory" received support in a subsequent investigation with the pyruvate oxidase system by WHITTAKER[5]. It is now generally assumed, though not proved, that a tervalent monosubstituted arsenical inhibits an enzyme by forming a cyclic thioarsenite with two juxtaposed sulfhydryl groups of the enzyme molecule, and that the dissociation of such a complex depends on the introduction of thiols having the property of forming more stable cyclic compounds with the arsenical.

The tervalent inorganic arsenical, arsenite, appears to possess certain properties in enzyme inhibition not shared by its organic counterparts. One of these is its dependence on a thiol for its inhibition. Several enzyme systems have been observed to require a thiol for arsenite inhibition. Examples are aldehyde dehydrogenases[6], oxidative phosphorylation[7], fatty acid biosynthesis[8], β-hydroxybutyrate dehydrogenase[9], acetyl-CoA carboxylase[10], and luciferase[11]. Most of these enzymes were also inhibited by Cd^{2+}. On the basis of these findings, the assumption that there are two juxtaposed sulfhydryl groups in these enzyme proteins has been made[6–11]. But the way in which the thiol mediates arsenite inhibition and the nature of the enzyme–inhibitor complex have not been investigated.

The preceding paper of this series[12] reported that glutamine synthetase (L-glutamate:ammonia ligase (ADP), EC 6.3.1.2) was inhibited by arsenite, but only in the presence of a certain dithiol. The enzyme was also inhibited by, among other metal ions, Cd^{2+}. The nature of arsenite inhibition of this enzyme has been studied in detail. Inhibition by organic arsenicals is also included for comparison. The results of these experiments form the subject of this presentation.

EXPERIMENTAL PROCEDURE

A partially purified glutamine synthetase preparation was obtained from rat liver as before[12]. This enzyme preparation was used in all experiments reported in this study, except where tissue and cell homogenates were studied. Glutamine synthetase activity was assayed as previously described[12], but with hydroxylamine concentration in the reaction mixture reduced from 44.5 mM to 8.9 mM. One unit of the enzyme activity is defined as the amount of enzyme that will cause the formation of 1 μmole of γ-glutamylhydroxamic acid in 1 h at 37°.

3-Amino-4-hydroxyphenyl arsenoxide hydrochloride (mapharside) interfered with the photometric determination of the hydroxamic acid, because it formed a deep orange color with the $FeCl_3$ reagent. When this compound was used in the experiments, the enzyme activity was determined by measuring the inorganic orthophosphate formed with the method of FISKE AND SUBBAROW[14]. The composition

of the reaction mixture and the conditions for incubation remained unchanged. At the end of incubation, however, 0.5 ml of 50% trichloroacetic acid was added to the 4.5 ml of the reaction mixture, and an aliquot of the mixture was used at once for the phosphate determination. The control contained ATP but no hydroxylamine. Both 2,3-dimercaptoquinoxaline and 3,4-dimercaptotoluene formed a black precipitate with the $FeCl_3$ reagent, but at a concentration of 0.22 mM of the dithiol, this precipitation did not interfere with the hydroxamic acid determination.

Protein was determined by the absorption at 280 mμ with a Zeiss PMQII spectrophotometer.

α,ω-Dimercaptoalkanes have low solubilities in water. Therefore, they were first dissolved in 60% isopropyl alcohol to give a 0.01 M solution. Aliquots of these solutions were then added to the reaction mixture. The highest concentration of the alcohol thus introduced into the reaction mixture was 13 μl/ml, which did not affect the enzyme activity. α,α'-Dimercapto-p-xylene and 3,4-dimercaptotoluene were added in a similar way.

All dithiols and arsenicals were commercial products. Dithiols were purchased from either Eastman Organic Chemicals or K & K Laboratories. p-Arsenosobenzoate was a product of Nutritional Biochemicals Corporation, and mapharside, of Parke, Davis and Company.

The detailed procedure for each experiment is given in the legend accompanying each table and figure.

RESULTS

Enzyme from different sources

The preceding paper of this series[12] has shown that the inhibition by arsenite (1.1 mM) of glutamine synthetase from rat liver depended on the presence of a dithiol, and that no inhibition occurred in the absence of an added thiol or in the presence of a monothiol. Therefore, an examination of the enzyme from other sources was made to determine the degree of dependence of arsenite inhibition on thiols. Table I shows these results. The inhibition of glutamine synthetase from a

TABLE I

DITHIOL-DEPENDENT ARSENITE INHIBITION OF GLUTAMINE SYNTHETASE FROM DIFFERENT SOURCES

Tissue or cell homogenates were used. The reaction mixture contained either no thiol or one thiol as noted below. Arsenite, when added ,was 1.1 mM. The inhibition is determined by comparing the enzyme activities in the presence and absence of arsenite.

Source of enzyme	*Per cent inhibition*		
	No thiol	*11 mM Cysteine*	*1.1 mM BAL*
Morris hepatoma 7800	1.8	2.1	76.9
Rat cerebrum	21.8	9.8	83.6
Chick cerebellum	6.4	0	29.1
Garter snake liver	3.2	0.8	81.2
Tetrahymena pyriformis	1.7	0	86.4
Neurospora crassa	23.4	12.1	95.0

rat hepatoma was much like that observed in rat liver. With the exception of the enzyme from chick brain, which was quite resistant to arsenite, the enzyme from other sources was inhibited to a similar degree by arsenite in the presence of BAL. One possible explanation for the sluggishness of arsenite inhibition in chick brain is the rapid oxidation of BAL by the brain homogenate. Although arsenite alone caused a significant inhibition of the enzyme from rat brain and Neurospora, BAL greatly enhanced the inhibition. On the other hand, cysteine, whose concentration was optimal for the enzyme activity, decreased arsenite inhibition. The decrease appears to be due to the formation of a thioarsenite, thereby reducing the concentration of the free inhibitor. The results, in general, demonstrate a similarity in arsenite inhibition of glutamine synthetase from widely diverse sources.

Comparison of dithiols

Experiments were then made with a number of dithiols to test their ability to mediate arsenite inhibition. Their effectiveness in fulfilling the sulfhydryl requirement of the enzyme is compared with that of BAL at the same concentration. Included is a homologous series of α,ω-dimercaptoalkanes. The results in Table II show that the relative effectiveness of the homologues in activating the enzyme decreased as the length of hydrocarbon chain between the two mercapto groups

TABLE II

RELATIVE EFFECTIVENESS OF DITHIOLS IN FULFILLING THE SULFHYDRYL REQUIREMENT OF GLUTAMINE SYNTHETASE AND IN MEDIATING ARSENITE INHIBITION

Dithiol (0.22 mM)	*Relative effectiveness*	*Per cent inhibition*	
		Arsenite (0.11 mM)	*Arsenite (0.22 mM)*
2,3-Dimercaptopropanol	(100.0)	38.4	63.0
1,2-Dimercaptoethane	96.4	51.9	63.7
1,3-Dimercaptopropane	65.8	21.6	43.4
1,4-Dimercaptobutane	65.8	2.2	26.1
1,6-Dimercaptohexane	62.9	0	0
1,8-Dimercaptooctane	62.1	2.4	12.2
1,10-Dimercaptodecane	56.1	0	2.2
3,4-Dimercaptotoluene	89.1	23.1	52.4
α,α'-Dimercapto-*p*-xylene	83.4	9.1	30.9
2,4-Dimercaptopyrimidine	34.8	0	0
2,3-Dimercaptoquinoxaline	54.6	0	2.8
None	39.7	0	0

increased. 1,2-Dimercaptoethane, for instance, showed an activity essentially the same as BAL, but 1,10-dimercaptodecane had little activity. In fact, the activity dropped sharply when the two mercapto groups changed from the 1,2- to the 1,3-positions. The ability of the homologues to mediate arsenite inhibition was examined at two concentrations of arsenite with the concentration of the dimercapto compounds maintained at 0.22 mM. The results also show that their ability to mediate arsenite inhibition depended on the proximity of the two mercapto groups in the molecule. 1,6-Dimercaptohexane and the higher homologues could not mediate the inhibition.

WHITTAKER[5] investigated the reactivation by α,ω-dimercaptoalkanes of pigeon-brain pyruvate oxidase after poisoning with lewisite and showed that 1,4-dimercaptobutane and 1,5-dimercaptopentane were less effective than their lower and higher homologues. Presumably, the requirement for stability of the arsenical–dithiol complex for mediation of an inhibition differs from that for reversal.

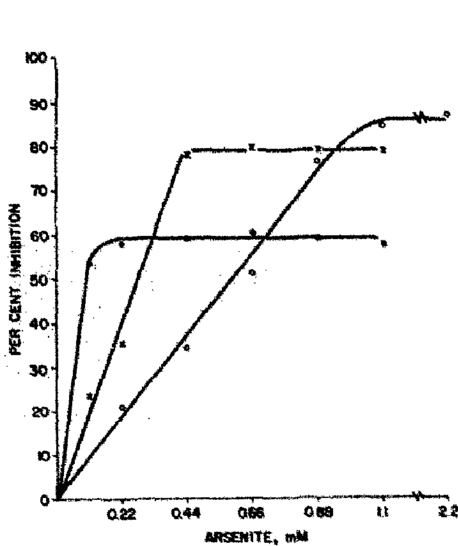
2,5-Dimercapto-1,3,4-thiadiazole already has been shown to be incapable of mediating arsenite inhibition[12]. Now a few other heterocyclic and aromatic dimercapto compounds have been investigated. Despite their closely placed mercapto groups, both 2,4-dimercaptopyrimidine and 2,3-dimercaptoquinoxaline had little or no activity and could not mediate arsenite inhibition. On the other hand, α,α'-dimercapto-*p*-xylene and 3,4-dimercaptotoluene exhibited significant activity toward the enzyme and to some extent mediated the inhibition. Hence, not all the dithiols that activate the enzyme can mediate arsenite inhibition. Neither the activity of a dithiol for the enzyme nor its ability to mediate the inhibition can be predicted solely from the proximity of the mercapto groups.

The partial requirement of glutamine synthetase for a thiol could be fulfilled by a much lower concentration of BAL than of a monothiol[15]. The requirement also depended on the age of the enzyme preparation. A freshly purified enzyme preparation had a lower requirement for a thiol than had a stored sample. Thus, the enzyme activity without a thiol varied from 30 to 50% of that with a thiol. But the degree of arsenite inhibition did not vary with the age of the preparation. Moreover, within the limits of the assay, doubling the enzyme concentration in the reaction mixture did not change the degree of arsenite inhibition.

In what follows, experiments will be described to elucidate the role of BAL in arsenite inhibition and the nature of the enzyme–arsenite complex.

Stoichiometric relation to BAL

FLUHARTY AND SANADI[7] have suggested that BAL serves as a vehicle for transporting arsenite to the sensitive site in the oxidative phosphorylating system. If this is true, low concentrations of BAL would be expected to be sufficient to mediate a full inhibition caused by high concentrations of arsenite, since one carrier molecule can transport repeatedly one arsenite molecule at a time to the sensitive site. The results in Fig. 1 show that, for a given concentration of BAL, the per cent inhibition increased with increasing concentration of the inhibitor, if the inhibitor concentration did not exceed the BAL concentration. Further increase in arsenite concentration beyond this limit resulted in no additional inhibition, unless, of course, BAL concentration was raised also. Clearly, availability of BAL limits the extent to which arsenite can inhibit. Furthermore, for a given concentration of arsenite, the greatest inhibition occurred when the molar ratio of BAL to arsenite was unity (Fig. 2). Increasing the ratio toward unity enhanced the inhibition, but further increase in the ratio beyond unity caused the inhibition to diminish. Most of the inhibition was prevented with the ratio approaching 2.5. These results show that the requirement for BAL in arsenite inhibition is stoichiometric and suggest that BAL serves as more than a vehicle for transporting the inhibitor to the sensitive site of the enzyme.



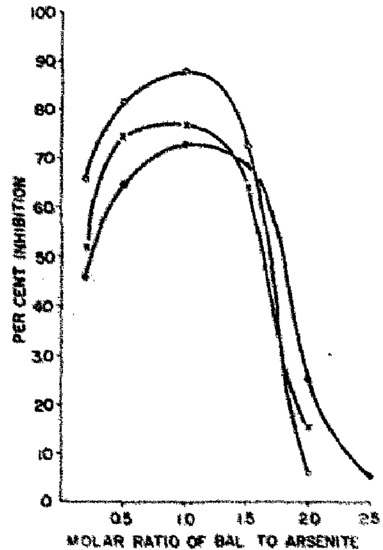


Fig. 1. Dependence of arsenite inhibition of glutamine synthetase on BAL. The figure shows arsenite inhibition in the presence of 0.11 mM (●—●), 0.44 mM (×—×), and 1.1 mM (○—○) BAL.

Fig. 2. Changes in arsenite inhibition of glutamine synthetase with the molar ratio of BAL to arsenite. The concentrations of arsenite in the reaction mixture were 0.44 mM (●—●), 0.66 mM (×—×), and 1.1 mM (○—○).

Inhibition by organic arsenicals

The degree of dependence of inhibition by organic arsenicals on a thiol has been studied for a comparison with arsenite inhibition. Fig. 3A shows that, in the absence of any thiol, both *p*-arsenosobenzoate and mapharside caused greater inhibition of glutamine synthetase than arsenite, and that mapharside was much more powerful than *p*-arsenosobenzoate. For instance, 0.022 mM mapharside caused about 40% inhibition as did 2.2 mM *p*-arsenosobenzoate or 8.8 mM arsenite. Arsenite

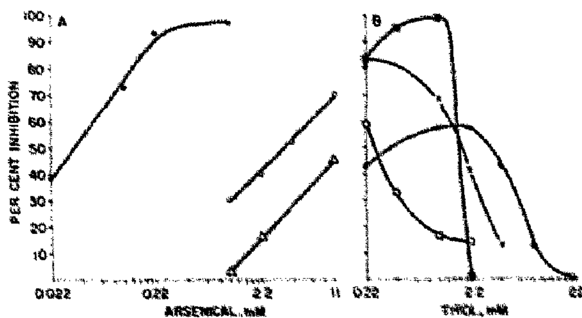


Fig. 3.(A) Inhibition of glutamine synthetase by mapharside (●—●), *p*-arsenosobenzoate (○—○), and arsenite (△—△) in the absence of a thiol. The concentration of the arsenical is expressed on a logarithmic scale. (B) Prevention of arsenical inhibition of glutamine synthetase by thiols. The graph shows changes in inhibition by 0.22 mM mapharside with cysteine (×—×) and with BAL (□—□), and by 2.2 mM *p*-arsenosobenzoate with cysteine (○—○) and with BAL (■—■).

could inhibit the enzyme without a thiol but only at high concentrations. BARRON *et al.*[16] have shown that arsenite was less effective in inhibiting sulfhydryl enzymes than organic arsenicals.

Although *p*-arsenosobenzoate could inhibit the enzyme without a thiol, the inhibition was intensified by a thiol, notably BAL (Fig. 3B). Excess thiol, however, prevented the inhibition completely. Cysteine enhanced the inhibition by *p*-arsenosobenzoate only to a limited extent, but BAL increased the inhibition to 100%. The greatest inhibition occurred when the molar ratio of BAL to *p*-arsenosobenzoate was 0.5; a ratio of one completely abolished the inhibition. With 0.22 mM mapharside, the inhibition was largely prevented when the molar ratio of BAL to the arsenical rose between 5 and 10. Cysteine was less effective than BAL in preventing inhibition by mapharside and by *p*-arsenosobenzoate. These results demonstrate some differences between the inhibition of glutamine synthetase by the organic arsenicals and that by arsenite. First, the inhibition by the organic arsenicals was not so much dependent on BAL as that by arsenite. Second, cysteine could increase the inhibition by *p*-arsenosobenzoate to some extent but not that by arsenite. In fact, 22 mM cysteine has been found to reverse the inhibition by 1.1 mM each of arsenite and BAL from 93.0 to 4.5%. Third, the molar ratio of BAL to the arsenical for maximum inhibition and for complete reactivation yielded different values for the three arsenicals.

Rate of inhibition

Fig. 4 shows the effect of prior incubation of glutamine synthetase with the arsenical on the extent of inhibition. Prior incubation was made in the presence of two substrates of the enzyme, the third substrate being added at the end to start

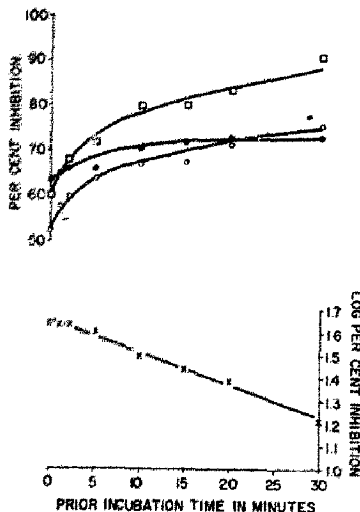


Fig. 4. Changes in inhibition of glutamine synthetase with time of prior incubation at room temperature by a mixture of 0.22 mM each of arsenite and BAL (●—●), by a mixture of 2.2 mM of *p*-arsenosobenzoate and 0.022 mM of BAL (○—○), by 0.66 mM of mapharside (□—□), and by 4.4 mM of *o*-iodosobenzoate (×—×). The per cent inhibition by *o*-iodosobenzoate is plotted on a logarithmic scale against time.

the reaction. Preliminary experiments showed that a prior incubation of the enzyme with the inhibitor in the presence of any one of the three substrates did not afford significant protection against the inhibitor. The results in Fig. 4 show that the inhibition by arsenite was almost complete immediately after its addition. The further increase in inhibition amounted to less than 10% before a plateau was reached after 15 min. of prior incubation. On the contrary, the inhibition by the organic arsenicals increased progressively with time of incubation throughout the entire period of observation. In all other experiments described in this study the prior incubation time of the enzyme with the arsenical was generally less than 5 min.

The inhibition of glutamine synthetase by *o*-iodosobenzoate decreased with the length of time of prior incubation. The decrease appears to be a spontaneous reactivation of the inhibited enzyme. The plot of logarithm of per cent inhibition against time gives a straight line (Fig. 4), thus showing that the reactivation follows first-order kinetics. However, since the enzyme used was only partially purified, the possibility that the decrease in inhibition may arise from a gradual inactivation of the inhibitor by foreign substances in the preparation cannot be excluded. ALDRIDGE[17] has documented one example of such a spontaneous phenomenon in the inhibition of cholinesterase by certain organic phosphorus compounds.

Effect of mercurial treatment on arsenite inhibition

STOPPANI *et al.*[18] have reported that mercurials afforded some protection against inhibition of yeast pyruvate decarboxylase by organic arsenicals. Therefore, the effect of mercurials on arsenite inhibition was examined. When glutamine synthetase was treated with *p*-chloromercuribenzoate or $HgCl_2$, the treated enzyme became sensitive to arsenite inhibition not only in the presence of BAL but also in the pre-

TABLE III

EFFECT OF PRIOR TREATMENT OF GLUTAMINE SYNTHETASE WITH MERCURIALS ON ITS INHIBITION BY ARSENITE IN THE PRESENCE OF CYSTEINE

0.5 ml of an enzyme preparation having 80 units of activity/ml was mixed with 0.4 ml distilled water and 0.1 ml of 1 mM *p*-chloromercuribenzoate or $HgCl_2$. The mixture was dialyzed against two changes of 500 ml each of 0.01 M Tris buffer (pH 7.2) for a total of 7 h. Aliquots of the dialyzed enzyme served for the following experiments.

Additions	*Relative activity*	*Per cent inhibition by arsenite*
A. *p*-Chloromercuribenzoate-treated		
None	5.2	
1.1 mM BAL	(100)	
1.1 mM BAL + 1.1 mM arsenite	11.0	89.0
2.2 mM cysteine	26.6	
2.2 mM cysteine + 1.1 mM arsenite	17.6	33.6
6.6 mM cysteine	46.5	
6.6 mM cysteine + 1.1 mM arsenite	26.6	42.8
B. $HgCl_2$-treated		
None	0	
11 mM cysteine	(100)	
11 mM cysteine + 1.1 mM arsenite	11.7	88.3

sence of cysteine (Table III). As has been shown earlier[12], monothiols could not mediate arsenite inhibition of the native enzyme. The effectiveness of cysteine in mediating arsenite inhibition could be manifested when the mercurial inhibition was partially reversed. But if excess cysteine was added to reverse the mercurial inhibition completely, a subsequent addition of arsenite did not produce an inhibition. Under these conditions, cysteine again could not mediate arsenite inhibition. One plausible explanation may be that inhibition in the presence of arsenite arises not from the action of arsenite on the enzyme but from the formation of a thioarsenite with cysteine, thereby decreasing the effective concentration of cysteine to reverse the mercurial inhibition. But the results in Table III also show that the inhibition in the presence of a given concentration of arsenite increased, rather than decreased, with increasing concentration of cysteine. This phenomenon parallels that observed with arsenite inhibition in BAL shown in Fig. 1. Therefore, the increase in inhibition in the presence of arsenite may indeed result from its direct action on the mercurial-treated enzyme. Possibly the mercurial treatment has resulted in a reversible change in the protein conformation in the vicinity of the sulfhydryl group with which arsenite reacts. In any case, the mercurial treatment has modified the dithiol-dependent inhibition by arsenite and has enabled a monothiol to mediate the inhibition.

Efforts to see whether treatment of the enzyme with guanidine hydrochloride or urea could produce an effect on arsenite inhibition similar to that of the mercurial treatment have been unsuccessful, owing to the sensitivity of the enzyme to these reagents. 1 M guanidine hydrochloride or 3.2 M urea caused a complete loss of the enzyme activity, which could not be restored by dialysis.

Glutamine synthetase was inhibited 47.5% and 86.0% by $6.7 \cdot 10^{-7}$ M and $6.7 \cdot 10^{-6}$ M Cd^{2+}, respectively. Prior treatment of the enzyme with Cd^{2+} at these concentrations was found to decrease arsenite inhibition subsequently. A detailed study of this interference, however, has not been made.

Dissociation of the enzyme–arsenite complex

The formation of a fairly stable ring structure by an arsenical with two jux-

TABLE IV

EFFECT OF DILUTION ON INHIBITION OF GLUTAMINE SYNTHETASE BY ARSENITE

0.8 ml of an enzyme solution containing about 32 units of activity was mixed with 0.2 ml of 0.01 M each of BAL and arsenite. The mixture was allowed to stand at 2° for 30 min and then diluted with distilled water in the proportions given below. About 15 min following the dilutions, 0.3-ml aliquots of the undiluted and diluted enzyme solutions were assayed for activity. Another 0.8 ml of the enzyme solution, similarly treated and diluted but with 0.2 ml distilled water replacing arsenite, served as a control. The specific activity of the enzyme in the control remained unchanged after the first two dilutions, but declined by about 20% after the next two higher dilutions. The specific activity of the inhibited enzyme increased progressively with dilution.

Condition	*Per cent inhibition*
Undiluted	50.9
Diluted 1:1	44.8
Diluted 1:2	39.6
Diluted 1:4	23.9
Diluted 1:9	19.2

taposed sulfhydryl groups in the protein molecule has been suggested to account for the inhibition of sulfhydryl enzymes by the arsenical[2,3,5]. Although the inhibition can readily be reversed by a dithiol, the reversal is assumed to take place because of a greater stability of the dithiol–arsenical complex than the enzyme–arsenical complex[3,16]. Hence, the inhibition is generally considered to be practically irreversible. STRAUS AND GOLDSTEIN[19] have shown that if an enzyme–inhibitor complex is not dissociable, then the fractional inhibition should not change upon diluting the complex. Otherwise, the complex would be dissociable. The results in Table IV show that the fractional inhibition of the enzyme–arsenite complex decreased progressively with dilution. Thus, a ten-fold dilution caused the inhibition to diminish from 50.9 to 19.2%. Further evidence for the dissociability of the complex has been obtained with gel filtration. Glutamine synthetase was excluded by Sephadex G-75. When it was filtered through the gel in a 7 × 1 cm column, the specific activity in the only fraction containing the enzyme activity decreased by 31.2% (Table V).

TABLE V

REACTIVATION OF ARSENITE-INHIBITED GLUTAMINE SYNTHETASE BY PASSAGE THROUGH SEPHADEX G-75 GEL

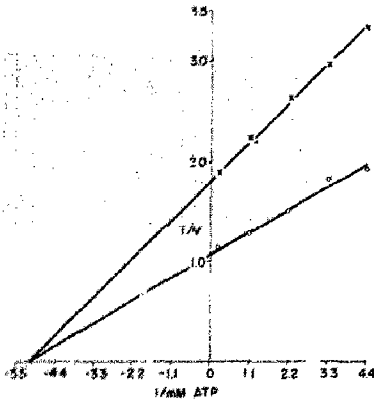
An enzyme preparation was mixed with BAL or an equimolar solution of BAL and arsenite, whose final concentrations were $4.2 \cdot 10^{-3}$ M. 0.5 ml of the mixture was allowed to pass through a 7 × 1 cm column of Sephadex G-75 gel previously equilibrated with 1 mM Tris buffer (pH 7.2). The enzyme was subsequently eluted with the same buffer. Only the second 2-ml fraction contained the enzyme activity; other fractions had no activity. The experiment was done at 5°. The enzyme activity was assayed without an added thiol.

Condition	*Before gel filtration*			*After gel filtration*			*Change in specific activity following filtration (%)*
	Protein (mg/ml)	*Enzyme activity (units/ml)*	*Specific activity (units/mg protein)*	*Protein (mg/ml)*	*Enzyme activity (units/ml)*	*Specific activity (units/mg protein)*	
Enzyme + BAL	2.1	94.4	44.0				
Second fraction*				0.45	13.9	30.9	−31.2
Enzyme + BAL + arsenite	2.1	60.0**	28.[illegible]				
Second fraction				0.45	14.0	31.1	+8.8

* The recovery values for total protein and enzyme activity of the uninhibited enzyme in this fraction gave 85.7% and 58.9%, respectively. The incomplete recovery of the enzyme activity appears to result, at least in part, from removal of BAL during the filtration, which lowers the enzyme activity. BAL was eluted in the fourth and fifth fractions.

** Arsenite caused 36.4% inhibition of the enzyme in this experiment. This rather low inhibition may arise from the lowering of the concentration of arsenite in the reaction mixture during assay to $5 \cdot 10^{-4}$ M — a dilution adequate to reduce the per cent inhibition (Table IV).

But when the arsenite-inhibited enzyme was similarly filtered, an increase of 8.8% was realized. Hence, gel filtration of the inhibited enzyme effected a relative increase of 40% in the specific activity when compared with that of the uninhibited enzyme. This increase appears to arise from removal of arsenite from the enzyme during gel filtration, since the amounts of protein excluded and the enzyme activities were the same in both cases. The two kinds of experiments described above give evidence for the dissociability of the enzyme–arsenite complex.



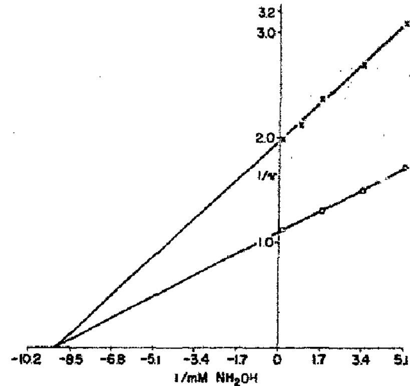


Fig. 5. Double reciprocal plot showing arsenite inhibition with respect to changes in ATP concentration. v denotes the number of μmoles of γ-glutamylhydroxamic acid formed in 15 min. The concentration of BAL was 0.11 mM in all experiments described in this and the following two figures. ×—×, with 0.11 mM arsenite; ○—○, without arsenite.

Fig. 6. Double reciprocal plot showing arsenite inhibition with respect to changes in hydroxylamine concentration. For details see the legend of Fig. 5.

Kinetics of inhibition

Because of the reversibility of the inhibiting reaction, the kinetics of arsenite inhibition has been studied in the usual way. The double reciprocal plots according to LINEWEAVER AND BURK[20] for each of the three substrates are shown in Figs. 5, 6, and 7. The apparent K_m values for L-glutamate, hydroxylamine, and ATP calculated from the plots gave $2.2 \cdot 10^{-3}$ M, $1.1 \cdot 10^{-4}$ M, and $2.0 \cdot 10^{-4}$ M, respectively. The values for hydroxylamine and L-glutamate are similar to those obtained with sheep-brain enzyme by PAMILJANS *et al.*[21]. But the value for ATP is one tenth of

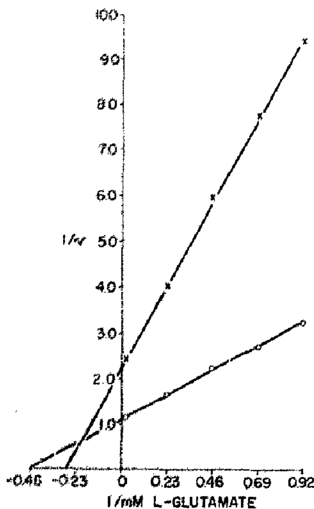


Fig. 7. Double reciprocal plot showing arsenite inhibition with respect to changes in L-glutamate concentration. For details see the legend of Fig. 5.

that with sheep brain enzyme. The inhibition by arsenite was non-competitive with respect to both hydroxylamine and ATP. The K_i values calculated from the graphs for hydroxylamine and ATP gave $1.5 \cdot 10^{-4}$ M and $1.9 \cdot 10^{-4}$ M, respectively. Hence the K_m values for these two substrates are essentially the same as the K_i. The graph for glutamate appears to represent a mixed type[22]. But since the intercept of the two lines was closer to the $-1/S$ axis than to the $1/v$ axis, the inhibition was probably non-competitive also.

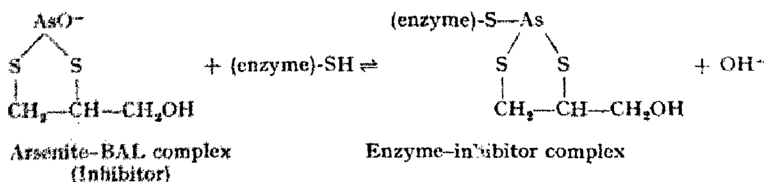
Perhaps the results obtained in the present study would have been more clean-cut, had a more highly purified enzyme preparation been used. Further investigations with suitable enzymes can yield more conclusive information concerning the mechanism of dithiol-mediated arsenite inhibition.

DISCUSSION

Two assumptions have been offered previously to describe the nature of arsenical inhibition. First, based on the nature of binding of lewisite to kerateine[2], arsenite was considered to form a similar ring structure with two juxtaposed sulfhydryl groups of the enzyme inhibited[3,6-11,18]. Second, perhaps as a corollary to the first assumption, arsenical inhibition was assumed to be essentially irreversible[3,18], that is, for all practical purposes, the enzyme-arsenical complex was not dissociable. The results of gel filtration and dilution experiments reported in this study invalidate the general applicability of the second assumption to arsenite inhibition of glutamine synthetase. The kinetic data further support the reversibility of arsenite inhibition, since K_i has been shown to be as high as K_m for hydroxylamine and ATP. In view of this evidence, the assumption that arsenite forms a relatively stable ring structure with glutamine synthetase seems untenable. If it does form a ring structure with the enzyme, the structure must be somewhat unstable and the As-S bonds readily broken. An alternative would be to assume that arsenite forms an open-chain complex with the enzyme. Although no evidence offering a distinct choice between the two alternatives has been obtained, the open-chain complex appears to be more consistent with the following observations. (a) Like other enzymes whose inhibition by arsenite is BAL-dependent[7-11], glutamine synthetase requires relatively high concentrations of arsenite for inhibition even when BAL is present. This may be compared with the classical example of pyruvate oxidase, which was inhibited 50% by about $2 \cdot 10^{-5}$ M of arsenite alone[1]. Possibly, then, arsenite does not react with two vicinal sulfhydryl groups in glutamine synthetase. (b) COHEN *et al.*[23] have shown the dissociability of the As-S bonds in R'-$As(SR)_2$. REISS AND HELLERMAN[24] have pointed out that if the formation of a thioarsenite involves only one thiol grouping, the complex may be considered spontaneously dissociable. Probably in the arsenite inhibition studied here, instead of a ring formation, arsenite forms a readily dissociable open-chain complex with only one sulfhydryl group of the enzyme.

A related question seems to be how BAL mediates arsenite inhibition so that in the end arsenite is bound to only one sulfhydryl group of the enzyme. Perhaps the question cannot be answered in a definitive way without intimate knowledge of the dissociation constants of the various complexes involved. But three points may be considered in order to formulate a probable mechanism for BAL mediation.

(a) The work of THOMPSON *et al.*[1-4] has shown that the reversal of lewisite or arsenite inhibition of pyruvate oxidase system by BAL is due to the formation of a more stable ring structure between BAL and the arsenical than that between the enzyme sulfhydryl groups and the arsenical. Furthermore, monothiols cannot reverse the inhibition because the open-chain thioarsenite formed between the monothiol and the arsenical is less stable than the enzyme–arsenical complex. (b) An 1:1 molar ratio of BAL to arsenite produced the greatest inhibition of glutamine synthetase at all concentrations of BAL and arsenite tested. (c) The arsenite–BAL complex, once formed, would be expected to enter the enzyme molecule as such. Indeed, it is very improbable that the two vicinal sulfhydryl groups (assumed present) of the enzyme could have a greater affinity for the As than BAL, so that the arsenite–BAL complex is broken, with the release of BAL and, at the same time, with the formation of a new ring structure. If this were the case, the enzyme–arsenite complex should have been more stable than the arsenite–BAL complex, and the inhibition could not have been reversed by BAL. The evidence described in this study is against the formation of such an indissociable enzyme–inhibitor complex. In view of these considerations, the following scheme is proposed for the BAL-mediated arsenite inhibition of glutamine synthetase:



Arsenite–BAL complex (Inhibitor) Enzyme–inhibitor complex

In the enzyme–inhibitor complex, the arsenic is bound to three S atoms. This structure resembles that found in tricysteinylarsine synthesized by JOHNSON AND VOEGTLIN[25]. The bond between the (enzyme)-S and As is like that formed by a monothiol with arsenite, and hence the complex would be readily dissociable. The nature of the complex also explains the requirement for equimolar quantities of BAL in arsenite inhibition. Furthermore, unlike the inhibition of pyruvate oxidase which cannot be reversed by cysteine, the dithiol-dependent inhibition of glutamine synthetase can be reversed by cysteine. The reversal may be looked upon as a displacement of the enzyme by cysteine in the complex; the nature of the (enzyme)-S–As bond permits such a displacement to take place. Finally, the formation of a relatively stable arsenite–dithiol complex with a five- or six-membered ring seems to be essential for inhibition, since 1,4-dimercaptobutane and the higher homologues mediated the inhibition poorly or not at all. One crucial test of the proposed scheme would be the ability to demonstrate the presence of the additional sulfhydryl groups contributed by BAL in the arsenite-inhibited enzyme after removal of BAL not bound to the enzyme. Efforts to obtain information in this respect have not been successful, owing to the high dissociability of the enzyme–inhibitor complex.

The foregoing postulation neither negates nor supports the view that glutamine synthetase may have a reactive disulfhydryl component[12]. The BAL–arsenite complex may act on either a single sulfhydryl group or two vicinal sulfhydryl groups. The inhibition of the enzyme by low concentrations of Cd^{2+} and mapharside, however,

does suggest the presence of two reactive sulfhydryl groups closely placed in the enzyme molecule. Presumably these sulfhydryl groups are oriented in such a way that arsenite alone is barred from approaching[7]. Based on the results presented in this study, a distinction would have to be made between the mechanism of inhibition by arsenicals, including arsenite, without the need for a dithiol and that by arsenite requiring a dithiol.

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REFERENCES

1 R. A. Peters, H. M. Sinclair and R. H. S. Thompson, *Biochem. J.*, 40 (1946) 516.
2 L. A. Stocken and R. H. S. Thompson, *Biochem. J.*, 40 (1946) 529.
3 L. A. Stocken and R. H. S. Thompson, *Biochem. J.*, 40 (1946) 535.
4 L. A. Stocken, R. H. S. Thompson and V. P. Whittaker, *Biochem. J.*, 41 (1947) 47.
5 V. P. Whittaker, *Biochem. J.*, 41 (1947) 56.
6 W. B. Jacoby, *J. Biol. Chem.*, 232 (1958) 89.
7 A. L. Fluharty and D. R. Sanadi, *J. Biol. Chem.*, 236 (1961) 2772.
8 R. O. Brady, E. G. Trams and R. M. Bradley, *Biochem. Biophys. Res. Commun.*, 2 (1960) 256.
9 I. Sekuzu, P. Jurtshuk, Jr. and D. E. Green, *J. Biol. Chem.*, 238 (1963) 975.
10 M. D. Hatch and P. K. Stumpf, *J. Biol. Chem.*, 236 (1961) 2879.
11 M. DeLuca, G. W. Wirtz and W. D. McElroy, *Federation Proc.*, 23 (1964) 103.
12 C. Wu, *Biochim. Biophys. Acta*, 89 (1964) 137.
13 C. Wu, *Arch. Biochem. Biophys.*, 106 (1964) 402.
14 C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, 66 (1925) 375.
15 C. Wu, *Comp. Biochem. Physiol.*, 8 (1963) 335.
16 E. S. G. Barron, Z. B. Miller, G. R. Bartlett, J. Meyer and T. P. Singer, *Biochem. J.*, 41 (1947) 69.
17 W. N. Aldridge, *Biochem. J.*, 54 (1953) 442.
18 A. O. M. Stoppani, A. S. Actis, J. O. Deferrari and E. L. Gonzalez, *Biochem. J.*, 54 (1953) 378.
19 O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, 26 (1943) 559.
20 H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658.
21 V. Pamiljans, P. R. Krishnaswamy, G. Dumville and A. Meister, *Biochemistry*, 1 (1962) 153.
22 J. L. Webb, *Enzymes and Metabolic Inhibitors*, Vol. 1, Academic Press, New York, 1963, p. 160.
23 A. Cohen, H. King and W. I. Strangeways, *J. Chem. Soc.*, (1931) 3043.
24 O. K. Reiss and L. Hellerman, *J. Biol. Chem.*, 231 (1958) 557.
25 J. M. Johnson and C. Voegtlin, *J. Biol. Chem.*, 89 (1930) 27.