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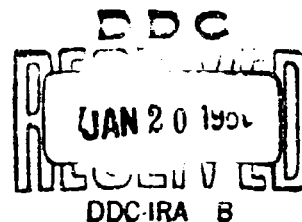
TECHNICAL MANUSCRIPT 261

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ENZYME DISTRIBUTION AS A FACTOR IN
THE INDEPENDENCE OF BACILLUS CEREUS
SPORE GERMINATION FROM L- AND
D-CYSTEINE DESULFHYDRASE ACTIVITY

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DECEMBER 1965



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ABSTRACT

The release of H_2S , pyruvate, and NH_3 from L- and D-cysteine by extracts from *B. cereus* strain T spores (0.2 M Tris, pH 8.0, 28 to 30 C in N_2 or air) is ascribed to the mediation of specific L- and D-cysteine desulphydrases. D-isomer activity is differentiated by pyridoxal phosphate independence, five-fold greater activity, and relative resistance to inhibition by semicarbazide. Inhibition by NH_2OH does not permit differentiation. Low recoveries of pyruvate (26 to 47%) are associated with a nonenzymic reaction between pyruvate and L- or D-cysteine. The distribution of the desulphydrases in the spore at some "deep" intrasporal sites that are internal to the locus of L-alanine dehydrogenase and a permeability barrier to L- and D-cysteine is postulated from the following: (i) L-cysteine-induced germination (0.2 M Tris, pH 8.0, 28 C, 1.0 mg/ml heat-shocked spores) and the specific stimulation of L-alanine-induced germination by the inactive inducing agent D-cysteine are independent of the desulphydrase activities: maximum rates of germination are observed when H_2S release during germination is inhibited (66 to 80%) by NH_2OH or semicarbazide. (ii) L-alanine dehydrogenase mediates the stimulation or induction of germination by cysteine and its activity precedes the expression of desulphydrase activity: inhibitors of L-alanine dehydrogenase inhibit H_2S release and germination described above completely but have little or no effect on cysteine desulphydrase activities of germinated spores. (iii) The desulphydrases are not surface enzymes: H_2S release is not evident in the absence of germination.

ENZYME DISTRIBUTION AS A FACTOR IN THE INDEPENDENCE
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The release of H_2S , pyruvate, and NH_3 from L- and D-cysteine by extracts from Bacillus cereus strain T spores is ascribed to the mediation of specific L- and D-cysteine desulphydrases (Fig. 1). The differentiation of two activities with optical specificity for the isomers of cysteine is evident when the release of H_2S from L- and D-cysteine is compared as a function of the concentration of pyridoxal phosphate. D-isomer activity is independent of the concentration of pyridoxal phosphate. Its activity, at one-fifth of the protein concentration employed in the determinations of L-isomer activity, is at least five-fold greater than that observed with L-cysteine. In contrast, L-isomer activity is markedly dependent on the concentration of pyridoxal phosphate, its activity increasing eight-fold with cofactor saturation. Dialysis for 60 hours against 5×10^{-4} M Tris pH 8.0 did not resolve a cofactor requirement for D-isomer activity. Both activities are heat-sensitive; 90% inactivation occurred in 15 minutes at 65 C and complete inactivation in 5 minutes at 100 C.

The desulphydrases are inhibited by the carbonyl reagents and pyridoxal phosphate antagonists NH_2OH and semicarbazide (Table 1). On the basis of concentration, NH_2OH is the more effective inhibitor. The inhibitory effect of semicarbazide, however, is of particular interest because it affords a further differentiation of the desulphydrases. The desulphydration of D-cysteine is less susceptible to semicarbazide inhibition, 5.0 mM semicarbazide eliciting only a 12% reduction in D-isomer activity as compared with a 53% reduction in L-isomer activity. The inhibitory effect of the pyridoxal phosphate antagonists on D-isomer activity may indicate that D-cysteine desulphydrase contains protein-bound pyridoxal phosphate that is not readily dissociable.

The formation of products H_2S , pyruvate, and NH_3 is consistent with desulphydrase activity and with the relative activities of the extracts toward the isomers of cysteine (Table 2). Marked discrepancies are apparent, however, in stoichiometry based on H_2S recovery: only 26 to 47% of the expected recovery of pyruvate was observed from L- and D-isomer activity; the recovery of NH_3 from L-isomer activity was 44% greater than anticipated. Since reasonable recoveries of NH_3 were obtained from D-isomer activity (75 to 84%), the discrepancy in NH_3 recovery from L-cysteine may be attributable to the presence of L-alanine dehydrogenase activity in our extracts. O'Connor and Halvorson* have reported that spores L-

* O'Connor, R.J.; Halvorson, H. 1961. The substrate specificity of L-alanine dehydrogenase. *Biochim. Biophys. Acta.* 48:47-55.

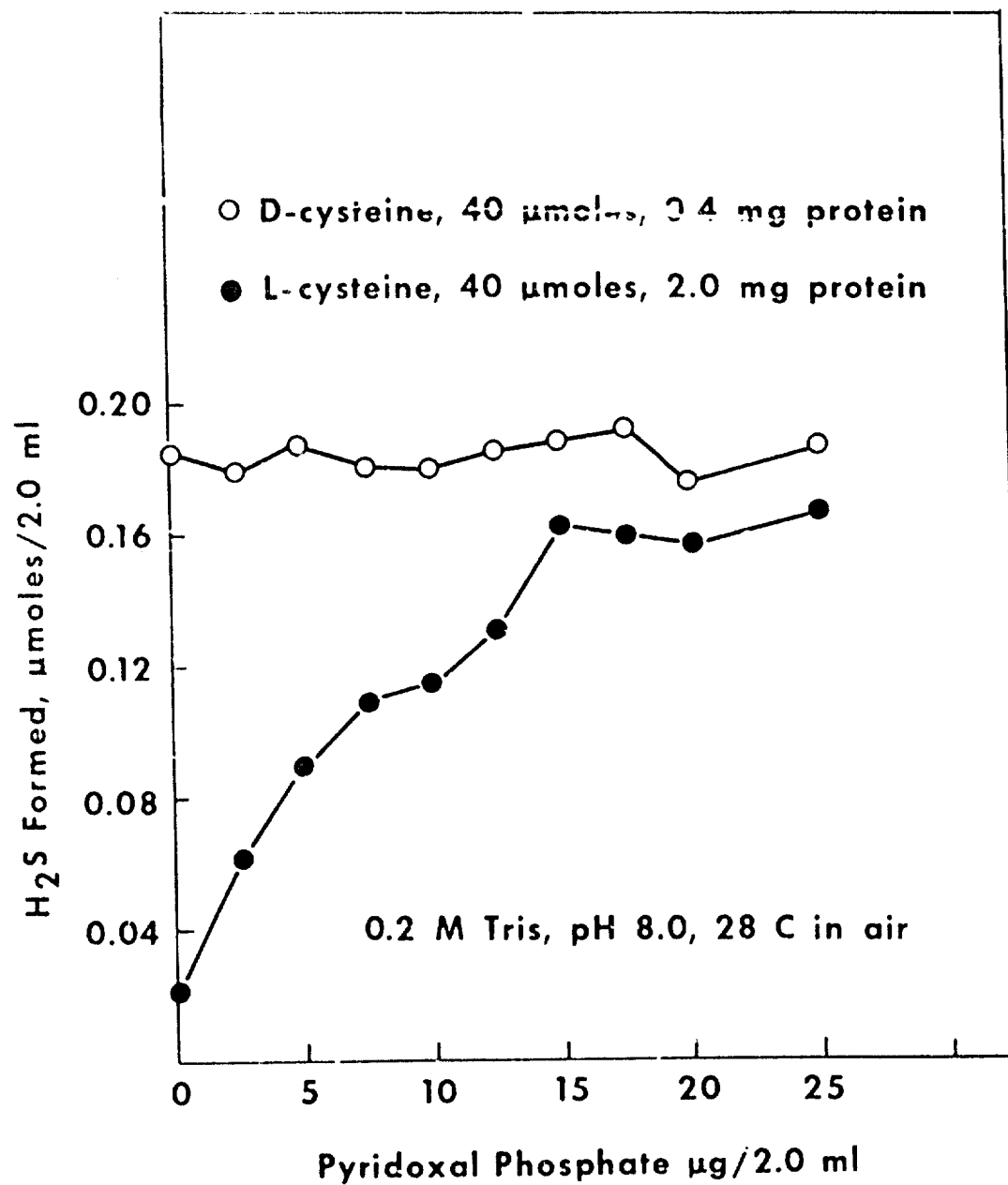


Figure 1. Effect of Pyridoxal Phosphate on the Evolution of H₂S from L- and D-Cysteine by Extracts from Bacillus cereus Strain T Spores.

TABLE 1. THE EFFECT OF NH_2OH AND SEMICARBAZIDE ON THE EVOLUTION OF H_2S FROM L- AND D-CYSTEINE BY EXTRACTS FROM BACILLUS CEREUS STRAIN T SPORES^a

Conditions	$\mu\text{moles H}_2\text{S}/2.0 \text{ ml}$		Per Cent Activity	
	L-cysteine	D-cysteine	L-cysteine	D-cysteine
<u>NH_2OH (mM)</u>				
0	0.183	0.206	100	100
0.005	0.139	0.169	75.8	81.4
0.01	0.120	0.114	65.5	55.0
0.02	0.104	0.074	56.9	35.6
0.05	0.060	0.035	32.8	16.9
0.15	0.024	0.013	13.1	6.3
<u>Semicarbazide (mM)</u>				
0	0.192	0.221	100	100
0.05	0.174	0.217	90.4	98.2
0.50	0.155	0.213	80.5	96.5
1.50	0.130	0.199	67.5	90.1
5.00	0.092	0.195	47.6	88.1
15.00	0.044	0.145	23.0	65.7

a. 0.2015 M Tris, pH 8.0 at 28 C in air.

TABLE 2. L- AND D-CYSTEINE UTILIZATION BY EXTRACTS FROM BACILLUS CEREUS STRAIN T SPORES^a

Conditions	$\mu\text{moles Product}/2.0 \text{ ml (molar ratio)}$					
	H_2S		Pyruvate		NH_3	
L-cysteine						
15 μmoles	0.82	(1.0)	0.21	(0.26)	1.18	(1.44)
D-cysteine						
15 μmoles	1.80	(1.0)	0.58	(0.32)	1.52	(0.84)

a. D-isomer activity at one-half protein for L-isomer; 0.203 M Tris, pH 8.0 at 30 C in N_2 .

alanine dehydrogenase deaminates the L-isomer of cysteine only. The low recoveries of pyruvate may be related to our observation (Table 3) that L- and D-cysteine react nonenzymically with pyruvate to form a compound that is not recoverable as a dinitrophenylhydrazone. The loss of pyruvate is a function of the concentration of L- or D-cysteine, an initial pyruvate to cysteine ratio of 1:10 resulting in an 80% loss of pyruvate. Although we have not identified the reaction product in our studies, its dependence on an alkaline pH for rapid formation and our inability to recover pyruvate with a reagent that reacts with an aldehyde group suggests that the reaction product is a thiazolidine carboxylic acid. It is of interest that Saz and Brownwell* have reported a pyridoxal phosphate independent D-cysteine desulfhydrase in Escherichia coli and have observed only 40% pyruvate recoveries in their product analysis.

The results of studies on the relationship of the desulfhydrases to germination demonstrate that the induction of germination by L-cysteine and the stimulation of L-alanine-induced germination by the inactive inducing agent D-cysteine are independent of L- and D-cysteine desulfhydrase activity. To explain the inability of these direct producers of pyruvate to induce germination, a process with a requirement for "internal pyruvate", we have postulated that the desulfhydrases are distributed in the spore at some "deep" intrasporal sites to which L- and D-cysteine are not accessible.

TABLE 3. THE NON-ENZYMIC LOSS OF PYRUVATE AS A FUNCTION OF THE CONCENTRATION OF L- OR D-CYSTEINE^a

L- or D-Cysteine, μmoles	Pyruvate Recovered, μmoles		Per Cent Recovery	
	L-cysteine	D-cysteine	L-cysteine	D-cysteine
0	4.88	4.86	97.7	97.3
10	2.74	2.98	54.8	59.6
20	1.88	1.92	37.7	38.5
30	1.44	1.44	28.9	28.9
40	1.20	1.24	24.0	24.8
50	1.06	1.10	21.2	22.0

a. 0.2 M Tris, pH 8.0 at 30 C in N₂.

* Saz, A.K.; Brownwell, L.W. 1954. D-Cysteine desulfhydrase in Escherichia coli. Arch. Biochem. Biophys. 52:291-295.

That the induction of germination by L-cysteine is independent of L-cysteine desulfhydrase activity is evident from the effect of semicarbazide and NH_2OH on germination and the release of H_2S (Fig. 2). Germination, expressed as the decline in optical density, and the release of H_2S during the course of germination were measured in 0.2 M Tris pH 8.0 at 28 C with 1.0 mg/ml of spores previously heat-shocked in water for 0.5 hours at 65 C. Semicarbazide inhibits the release of H_2S during the course of germination but has no effect on the induction of germination by L-cysteine. As is evident from the identical optical density decline in the absence and presence of semicarbazide, the rate of L-cysteine-induced germination is independent of the rate of release of H_2S . Similar results were observed with NH_2OH (Fig. 3). Reductions of as much as 80% of the H_2S released during the course of germination had no effect on the rate of germination.

With respect to the utilization of D-cysteine, prior studies* have shown that D-cysteine is inactive as an inducing agent. D-cysteine, however, specifically stimulates both the rate and extent of germination initiated by L-alanine. The specific stimulation of L-alanine-induced germination was explained by the demonstration that D-cysteine inhibits spore alanine racemase.

That the stimulation of L-alanine-induced germination by D-cysteine is independent of D-cysteine desulfhydrase activity was demonstrated in studies with NH_2OH (Fig. 4). L-Alanine in the absence or presence of NH_2OH initiates only a slight optical density decline. In the presence of D-cysteine with and without NH_2OH a like concentration of L-alanine initiates rapid and complete germination. The reduction of two-thirds of the H_2S release during L-alanine-induced germination with both D-cysteine and NH_2OH , however, is without effect on the rate of germination. The independence of the stimulatory effect of D-cysteine on L-alanine-induced germination from the release of H_2S demonstrates that D-cysteine desulfhydrase activity makes no contribution to germination. Semicarbazide and NH_2OH thus in effect uncouple L- and D-cysteine desulfhydrase activity from the induction processes of spore germination.

* Krask, B.J. 1961. Discussion, p. 89-100. In H.O. Halvorson (ed.), Spores, Vol. II. Burgess Publishing Co., Minneapolis.

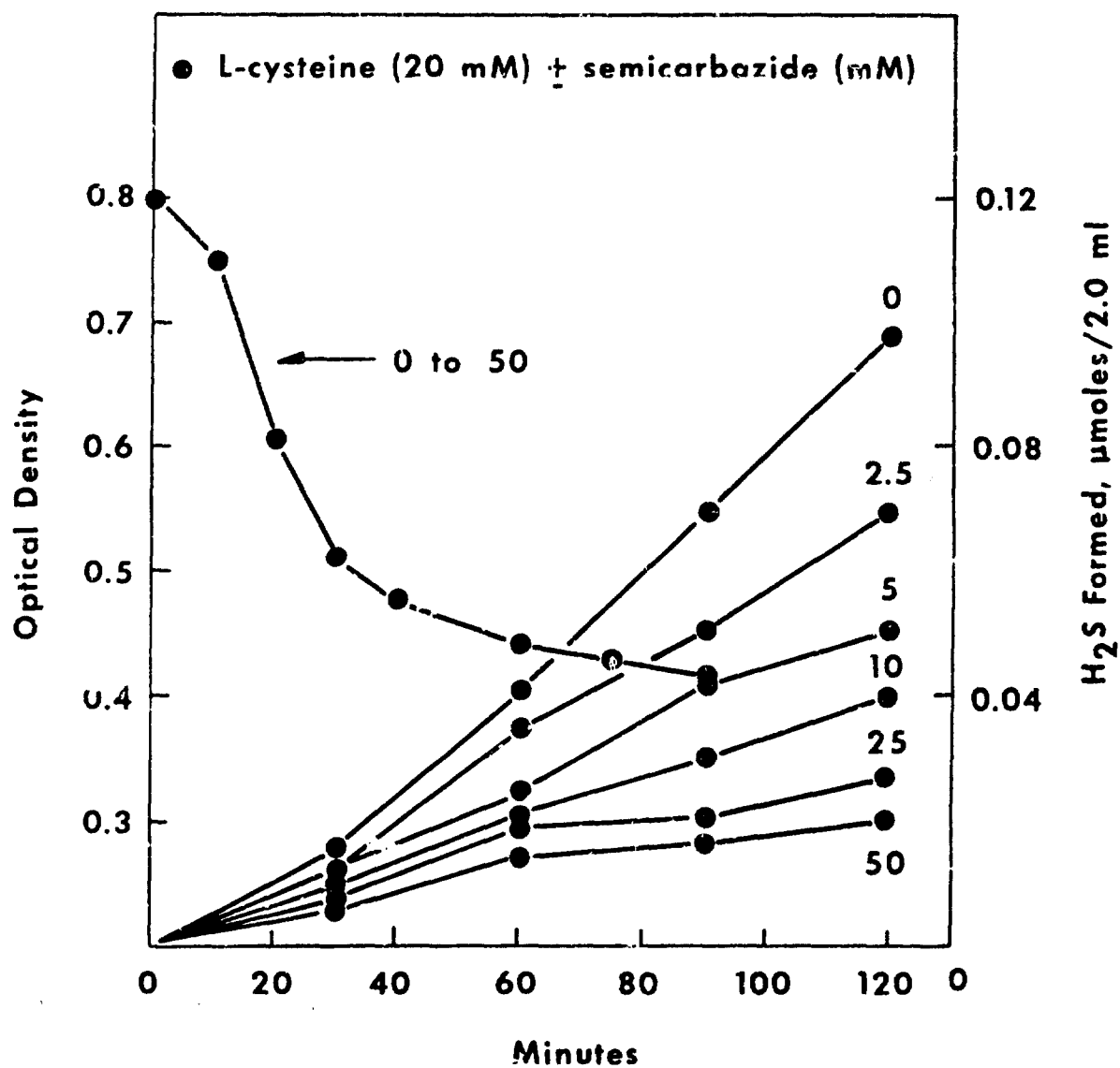


Figure 2. Effect of Semicarbazide on the Rate of L-Cysteine-Induced Germination and L-Cysteine Desulphydrase Activity of *Bacillus cereus* Strain T Spores.

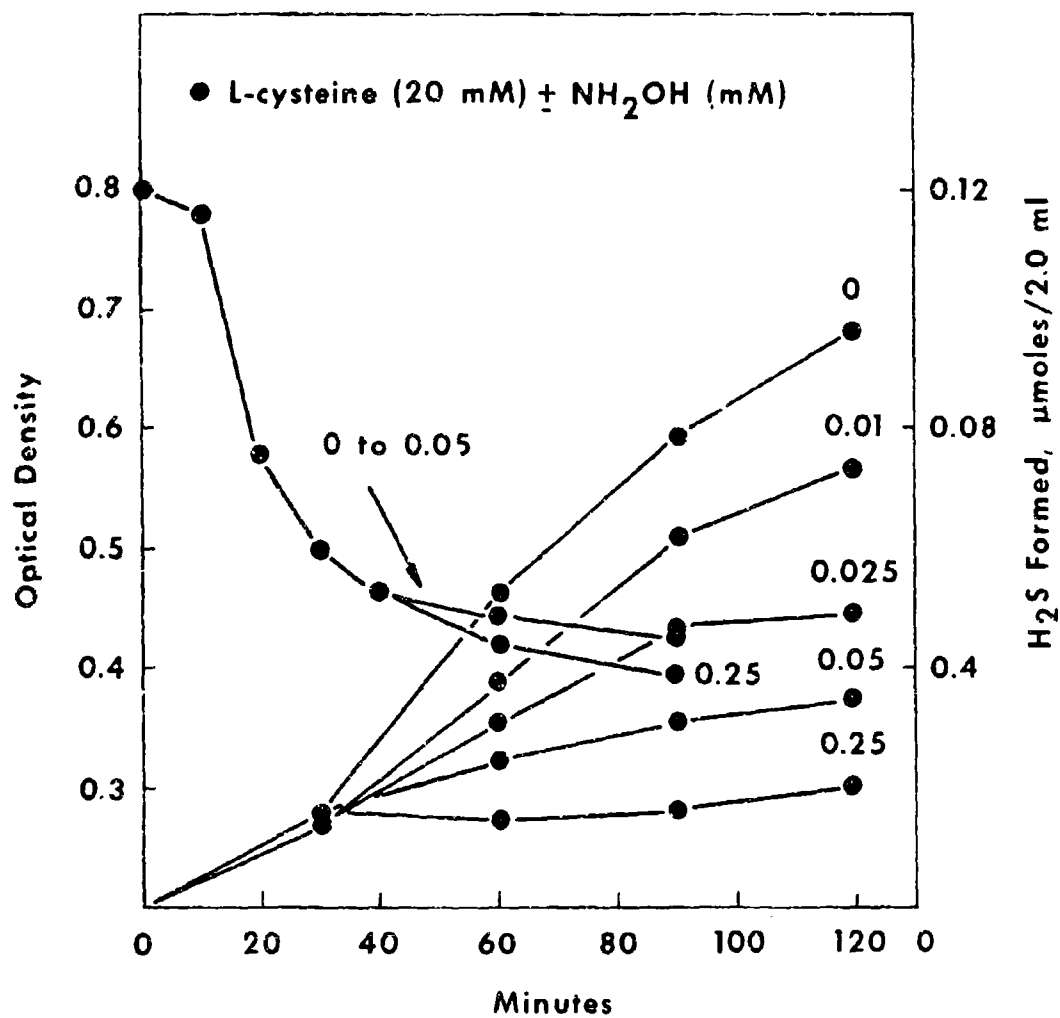


Figure 2. Effect of NH₂OH on the Rate of L-Cysteine-Induced Germination and L-Cysteine Desulphydrase Activity of Bacillus cereus Strain T Spores.

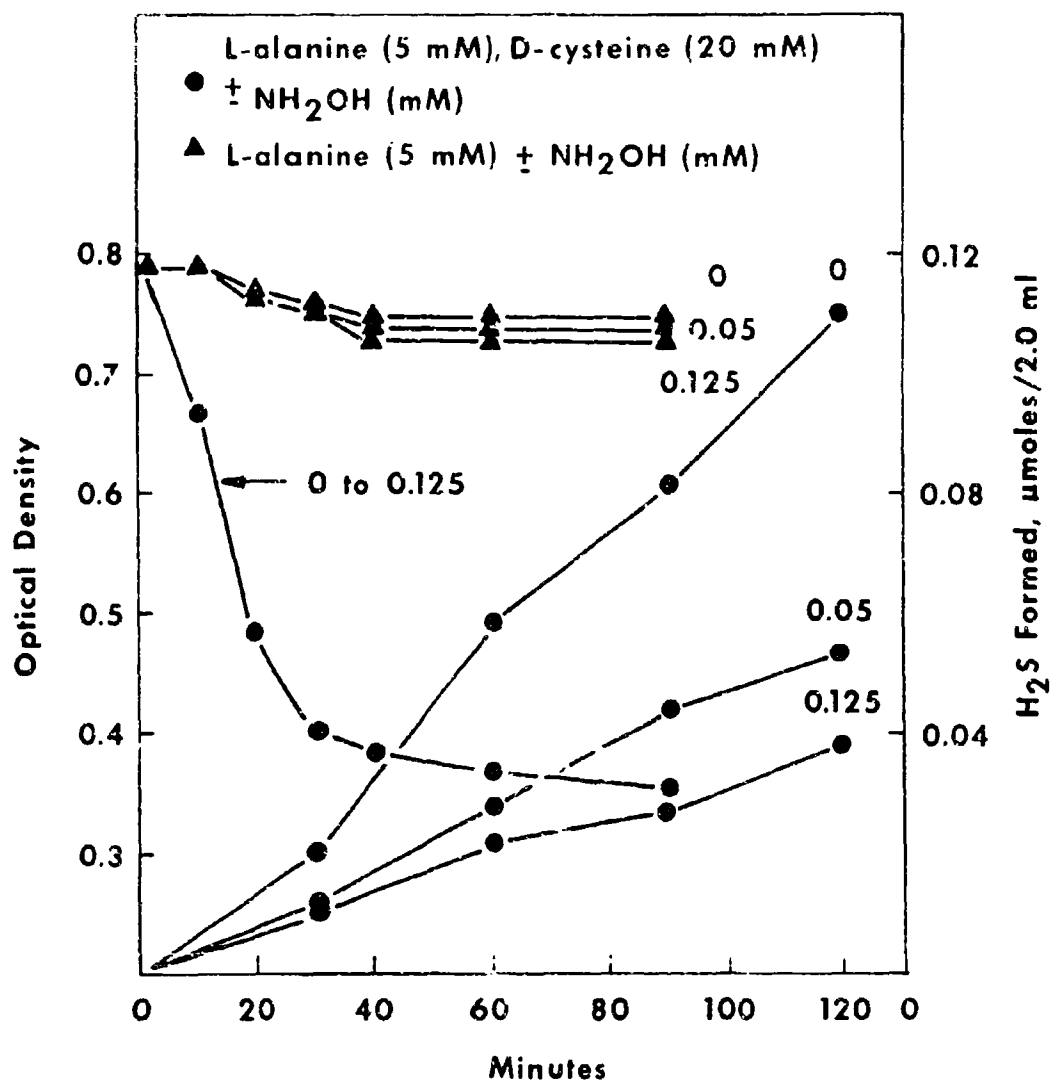


Figure 4. Effect of NH_2OH on the Rate of L-Alanine-Induced Germination in the Presence of D-Cysteine and on D-Cysteine Desulfhydrase Activity of *Bacillus cereus* Strain T Spores.

The uncoupling of the desulphydrase activities from a role in the induction of germination was confirmed by the demonstration that the induction of germination, presumably by L-alanine dehydrogenase activity, must precede the expression of L- and D-cysteine desulphydrase activity. That the release of H_2S occurs only after the spore has germinated is evidenced by the demonstration that the inhibitors of L-alanine dehydrogenase, D-alanine, glycine, and D- α - NH_2 -butyric acid, inhibit L-cysteine-induced germination and the release of H_2S , although they have little effect on L-cysteine desulphydrase activity of germinated spores. The decline in optical density reflecting the induction of germination by 20 mM L-cysteine (Fig. 5) is completely inhibited by 2.5 mM D-alanine and 10 mM glycine and is markedly affected by 25 mM D- α - NH_2 -butyric acid. Similarly, the release of H_2S during germination is completely inhibited or markedly reduced at the concentrations of the D-amino acids and glycine that produced a complete inhibition of germination. Reductions in the concentrations of the inhibitors of L-alanine dehydrogenase are reflected in paralleled but lessened inhibitory effects on the rates of germination and the rates of release of H_2S . D-Alanine and glycine similarly inhibit germination and H_2S release during L-alanine-induced germination in the presence of D-cysteine (Fig. 6).

That the inhibitory effect of the D-amino acids and glycine on germination and H_2S release is unrelated to the inhibition of the desulphydrase activities is evident from their relative inability to effect the release of H_2S from L- and D-cysteine by germinated spores. D-alanine, at a concentration eight-fold greater than that required to inhibit L-cysteine-induced germination and H_2S release completely, reduces L-cysteine desulphydrase activity of germinated spores to a maximum of 13%; glycine and D- α - NH_2 -butyric acid effect reductions of 13% and 21% respectively (Fig. 7). Similarly, D-alanine and glycine have little effect on D-cysteine desulphydrase activity of germinated spores (Fig. 8).

Because the inhibitory effect of the D-amino acids and glycine on germination and H_2S release is unrelated to the inhibition of L-cysteine desulphydrase activity, our results suggest that L-cysteine-induced germination is mediated by L-alanine dehydrogenase and the choice of mediator is determined by the relative distribution of L-alanine dehydrogenase and L-cysteine desulphydrase within the spore. The inability of the spore to release H_2S in the absence of germination demonstrates that L-cysteine desulphydrase is not a surface enzyme. The necessity for the induction of germination to precede the expression of L-cysteine desulphydrase activity suggests that the locus for L-cysteine desulphydrase in the spore is internal to that of L-alanine dehydrogenase. A locus for the desulphydrase at the level of L-alanine dehydrogenase is precluded because H_2S release was not observed during the complete inhibition of germination by the inhibitors of L-alanine dehydrogenase. Since the spore is obviously permeable to L-cysteine at the locus of L-alanine dehydrogenase in the spore, it must not be permeable at some level that precedes the locus of L-cysteine desulphydrase. Our observations with D-cysteine similarly suggest that D-cysteine desulphydrase is distributed within the spore at some "deep" intrasporal site which is internal to L-alanine dehydrogenase.

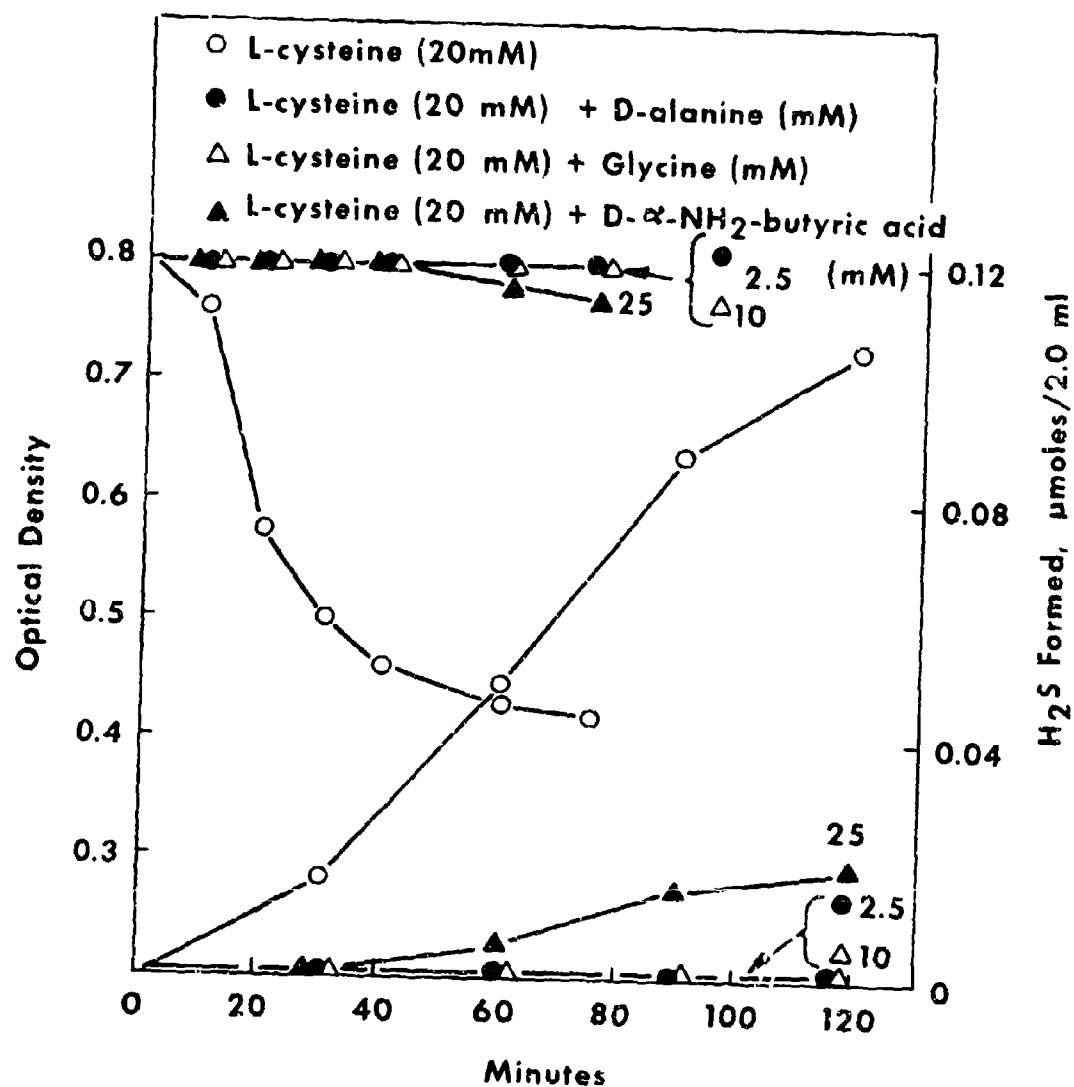


Figure 5. Effect of Inhibitors of L-Alanine Dehydrogenase on L-Cysteine-Induced Germination and L-Cysteine Desulfhydrase Activity of *Bacillus cereus* Strain T Spores.

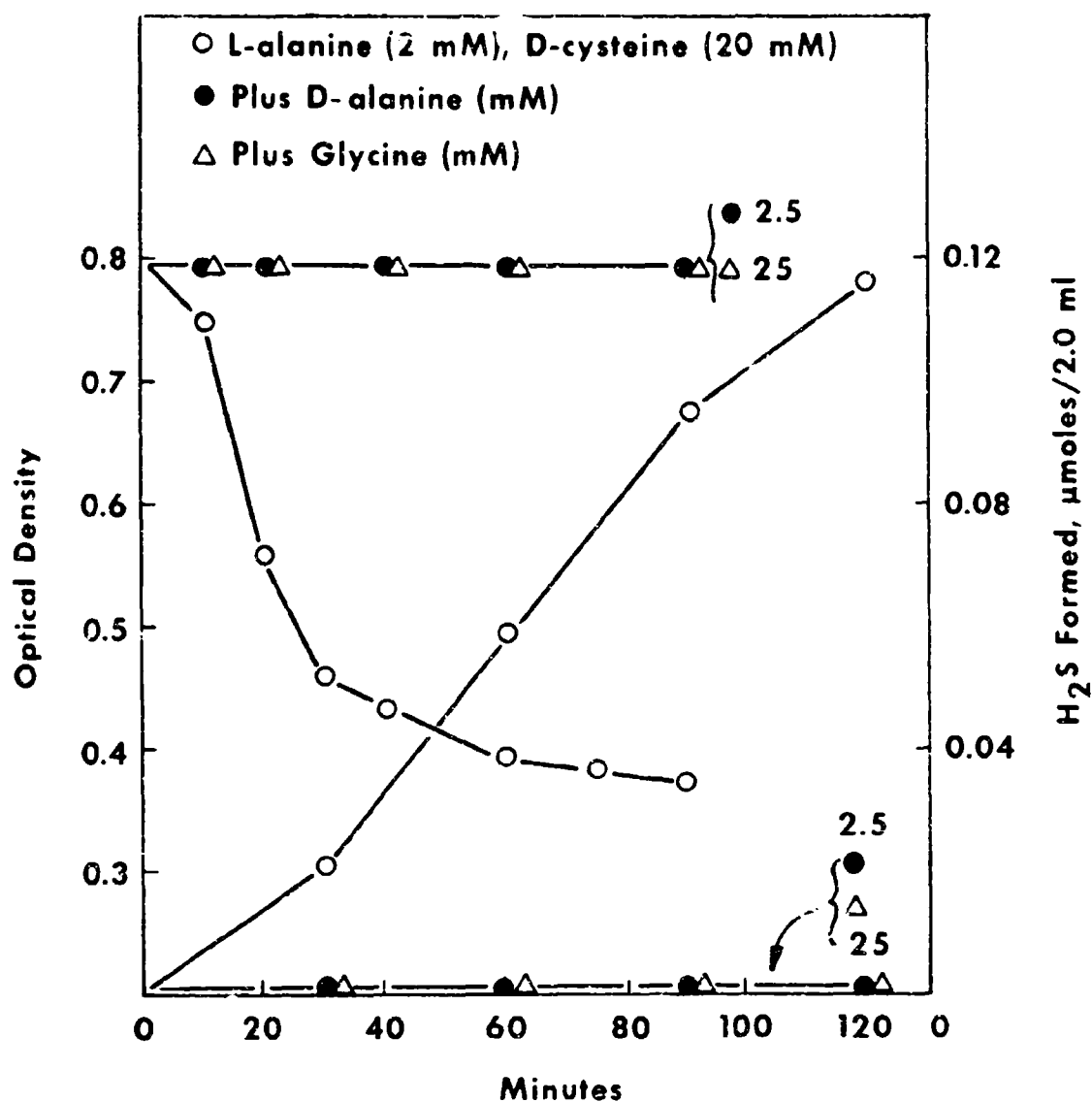


Figure 6. Effect of Inhibitors of L-Alanine Dehydrogenase on L-Alanine-Induced Germination in the Presence of D-Cysteine and on D-Cysteine Desulphydrase Activity of Bacillus cereus Strain T Spores.

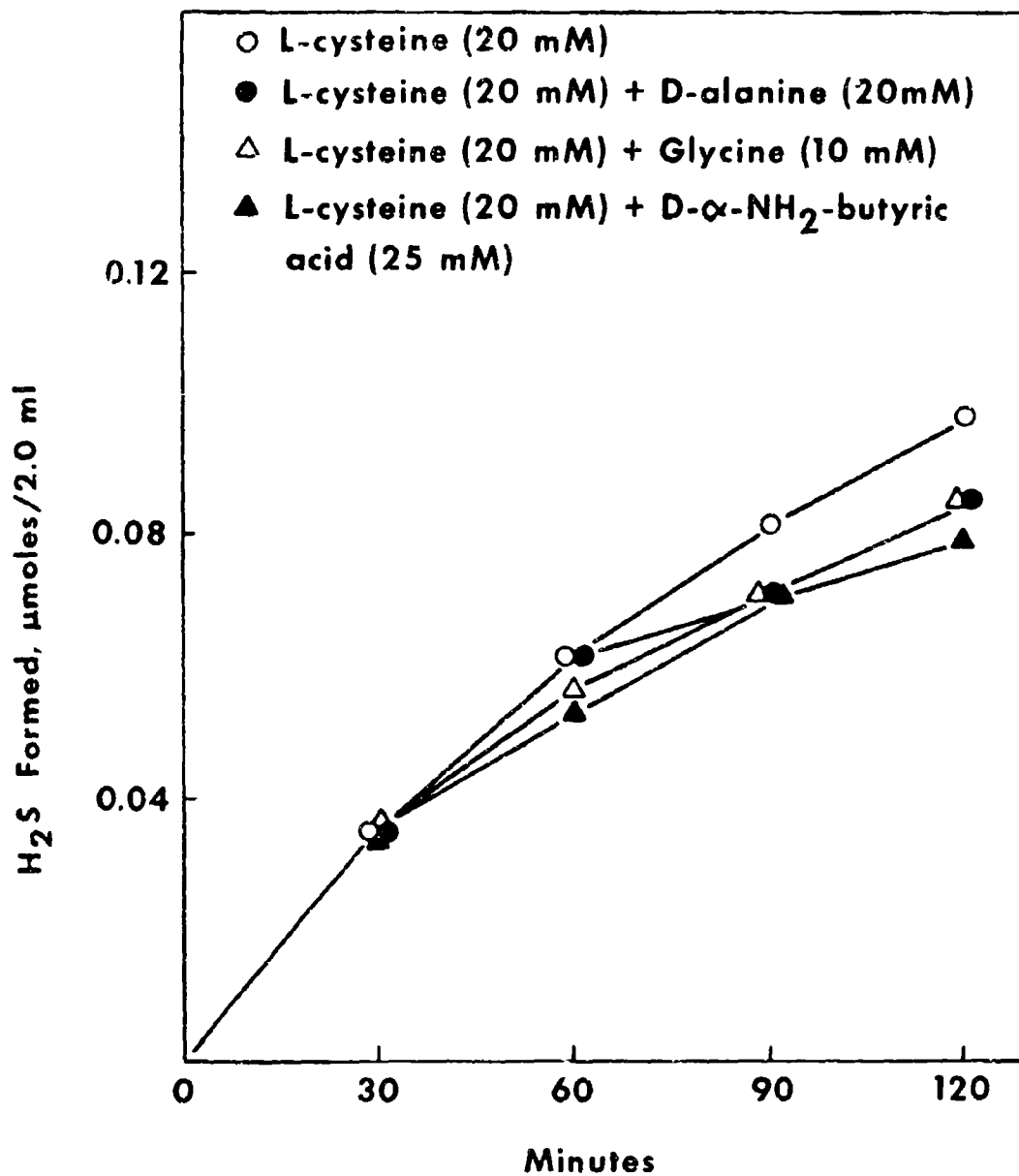


Figure 7. Effect of Inhibitors of L-Alanine Dehydrogenase on L-Cysteine Desulfhydrase Activity of Germinated Spores of *Bacillus cereus* Strain T.

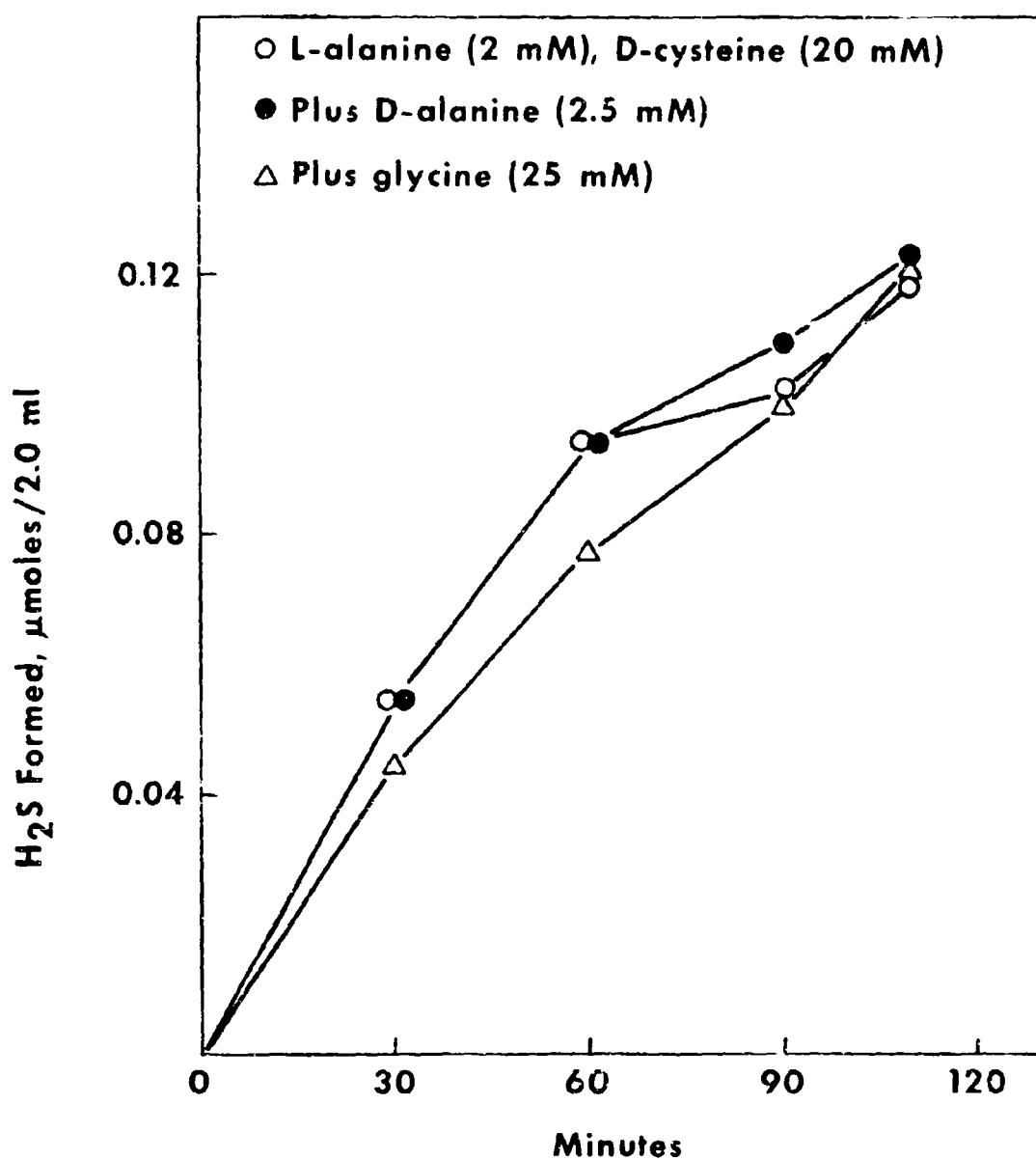


Figure 8. Effect of Inhibitors of L-Alanine Dehydrogenase on D-Cysteine Desulphydrase Activity of Germinated Spores of Bacillus cereus Strain T.

Because germination precedes the expression of the desulfhydrase activities and the swelling of the spore which accompanies germination may be related to the expansion of the "contractile cortex," it is attractive to speculate that the distribution of the desulfhydrase in the spore may be intracortical or within the core of the spore. The "contracted cortex" condition of the spore prior to germination could maintain intracortical desulfhydrase in a refractory state through compression of secondary protein structure such that the active sites of the enzymes were not accessible to cysteine. Alternatively, the contracted cortex could exclude L- and D-cysteine from desulfhydrase distributed in the core of the spore.