ACTIVATORS FOR THE CYSTEINE DESULFHYDRASE SYSTEM OF AN ESCHERICHIA COLI MUTANT

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The literature concerning the microbial degradation of cystine has been reviewed recently by Fromageot (1951) and by Kallio and Porter (1950). The studies of the latter workers with the cysteine desulfhydrase systems of *Proteus morganii* and *Proteus vulgaris* showed the anaerobic dissimilation of cystine to cysteine with the subsequent breakdown of cysteine to pyruvate, ammonia, and hydrogen sulfide. Among other pertinent observations they observed that toluene-treated cells of *Proteus morganii* "aged" at pH 4.5 were considerably inactivated with respect to desulfhydrase activity, and that the activity could be partially restored by the addition of boiled cell extracts, but not by the addition of vitamins, divalent ions, or combinations of glutathione with adenosine-3-phosphate or adenosine-5-phosphate. Of interest and importance to the present study is the finding of Wood and Gunsalus (1949) that purified serine and threonine deaminase from *Escherichia coli* were inactive on cysteine, thus presenting evidence against the identity of the dehydrase (deaminase) as the desulfhydrase as had been previously indicated (Binkley, 1943). It should be noted that Binkley's findings do not constitute evidence for identicalness nor is a claim for such made. The suggested competitive nature of the activity of serine or phosphoglyceric acid when added to the cell-free extract is explainable on the basis of structural similarity of the molecules and resulting competition for the cysteine desulfhydrase.

Since the anaerobic elimination of hydrogen sulfide by desulfhydrase activity and the removal of water by dehydrase (deaminase) activity are basically similar chemical processes, it is of interest to study the cofactor requirements for the desulfhydrase with the intent of ascertaining whether or not similarities exist. Positive findings in this respect would lead to the expectation of similar coenzyme requirements for similar type reactions without specific identity of functional groups being required. This concept is not unique since a similar type of activity occurs in the replacement of phosphate with arsenate in certain glycolytic transformations, and can doubtless be shown elsewhere. An example would be provided, however, of this sort of phenomenon occurring in normal processes. This investigation concerned this type of a similarity and reports cofactor requirements for desulfhydration in *E. coli* with an interpretation of the data in terms of a desulfhydration and direct removal of ammonia by hydrolytic cleavage of the primary desulfhydration product or a tautomer. The finding of a pyridoxal phosphate stimulation by Kallio (1951) in *Proteus* was readily confirmed in this laboratory in *E. coli* and suggested to us that the stimulation noted may be a reflection of a linked transaminase operating *via* removal of alanine

resulting from reduction of either aminoacrylic acid or iminopropionic acid. Our results lead us to believe that the function is more direct, and that the pathway to pyruvate, ammonia, and hydrogen sulfide is *via* the long postulated direct route. (For a discussion of mechanism see Fromageot, 1951.)

METHODS

Most of these studies were made with a biotin-requiring strain of *E. coli*, which was very active in cysteine degradation. The biotin-requiring characteristic is not to be considered pertinent to the data or to its interpretation since adenosine-5-phosphate stimulation could be shown with the well-known Crookes strain as well as with this organism. Furthermore, it is obvious that the culture medium employed is adequate in biotin content, yet cells harvested from it are still stimulable by the adenylic acid as well as by the other activators described. It is therefore considered that the activations observed are not directly related to the biotin-requiring characteristic of the mutant.

Cells were produced by a 20-hour incubation at 37 C after a 10 per cent inoculation into a 1 per cent tryptone medium containing 0.12 per cent K_2HPO_4, and 0.5 mg per ml of cysteine hydrochloride added aseptically after sterilization. The final pH of the medium was adjusted to be in the vicinity of pH 5.8 to 6.0 after the addition of the cysteine. After harvest by centrifugation, cells from 4 liters of medium were resuspended in 10 ml of distilled water, allowed to age at room temperature for 1 hour, washed twice with 10 ml H_2O, subjected to sonic vibration until microscopic examination revealed almost complete disruption, and then centrifuged at high speed. The desulfhydrase activity was found in the debris fraction with an almost complete absence of activity in the supernatant. The active fraction was then suspended in 20 to 30 ml of distilled water and tested for desulfhydrase activity. An index of cell concentration was made by turbidimetric measurement of the suspension before vibration. Cell disruption was accomplished in a Raytheon 9KC Magnetostriction Oscillator with the plate voltage set at 80 volts. The time of vibration was variable, 10 to 30 minutes being the time necessary, usually, for complete disruption. The degree of disruption varied with different cell lots, perhaps as a result of varying energy output of the instrument, hence microscopic examination from time to time throughout the vibration procedure was necessary. Excessive vibration resulted in complete inactivation of the desulfhydrase system.

Desulfhydrase activity was measured by incubation of the cell preparation with cysteine in the presence of phosphate buffer and various activators with pH control accomplished by the use of appropriate buffer and proper neutralization of test substances where required. The endogenous activity of the vibrated cell preparations was nil. At the termination of the experiment the reactions were stopped by the addition of 2 drops of 40 per cent NaOH. The use of trichloracetic acid was unsatisfactory because of an interference with the color and stability of the subsequently measured lead sulfide. Cell material was removed by high speed centrifugation, and an aliquot of the supernatant was withdrawn for sulfide analysis. Aliquots up to 4.0 ml in amount were added directly to a 10 ml colorim-

eter tube which contained 2.0 ml of 2 M NaOH. The nonorganic sulfide present was precipitated as colloidal lead sulfide by the addition of 4.0 ml of an aqueous reagent containing 1.0 g of crystalline lead acetate, 2.5 ml of glacial acetic acid,

TABLE 1

Effects of various metabolities on the cysteine desulfhydrase system

	ADDITIONS	Q_{H_2S} (N)*
(Experiment III-54, cell-free, pH 7.1)	None	9
	Adenosine-5-phosphate	18
	Biotin	11
	Pyridoxal-phosphate	18
	Alpha-ketoglutarate	13
	Adenosine-5-phosphate, biotin	19
	Adenosine-5-phosphate, pyridoxal phosphate	38
	Adenosine-5-phosphate, *alpha*-ketoglutarate	19
	Biotin, pyridoxal phosphate	29
	Biotin, *alpha*-ketoglutarate	23
	Pyridoxal phosphate, *alpha*-ketoglutarate	40
	Adenosine-5-phosphate, biotin, pyridoxal phosphate	44
	Adenosine-5-phosphate, biotin, *alpha*-ketoglutarate	24
	Adenosine-5-phosphate, pyridoxal phosphate, *alpha*-ketoglutarate	45
	Biotin, pyridoxal phosphate, *alpha*-ketoglutarate	40
	Biotin, pyridoxal phosphate, *alpha*-ketoglutarate, adenosine-5-phosphate	53
(Experiment III-56, cell-free)	None	36
	Glutamic acid	36
	Aspartic acid	32
	Pyruvic acid	43
	Semicarbazide	13
	Alanine	26
	Serine	29
	Phosphoglyceric acid	27
(Experiment III-78, cell-free)	None	18
	Alanine-glutamic transaminase (crude)	19
	Alanine-glutamic transaminase "kochsaft"	28
(Experiment IV-88, cell-free)	Biotin, *alpha*-ketoglutarate, adenosine-5-PO_4	38
	Plus added alanine-glutamic transaminase (8 units)	54
	Plus added alanine-glutamic transaminase "kochsaft" (from 8 units) (no active transaminase)	56

*Q_{H_2S} (N) = μg H_2S per mg cell H per hr.

and 2.5 g of gum arabic per liter. An immediate colloidal precipitate of lead sulfide resulted which was stable for at least 15 minutes, and which could be read in the colorimeter. Hydrogen sulfide was determined by direct reference to a standard curve prepared by using crystalline lead acetate as a primary standard and a solution of sodium sulfide as a precipitating agent. The standardization

procedure required the omission of the lead acetate from the assay reagent, its addition in known amounts to the standardization tubes, and the addition of excess sulfide as the last addition before making to volume with water. In the preparation of the standard lead acetate solution a few drops of glacial acetic acid were included to eliminate turbidity resulting from hydrolysis. This indirect procedure was required because of the poor characteristics of the soluble metallic sulfides as primary standards. A standard curve prepared in this manner with absorption (and scattering) measured at 490 mμ resulted in an almost linear relationship when a log function of the galvanometer reading was plotted against sulfide concentration. Hydrogen sulfide could be estimated in concentrations from 3 to 100 μg with an absolute error not exceeding ±2 μg. Control tests without the addition of cell preparations consistently were completely negative with respect to chemical sulfide production from cysteine under the conditions of the analyses, thus assuring no analytical complications due to the relative instability of cysteine.

Activity of the cell preparations was determined by test tube incubation at 37 C in a total volume of 4.5 ml with a weak phosphate buffer concentration in order to minimize salt inhibition. The incubation medium was prepared by the addition of various activators (table 1) to 1.0 ml of M/10 phosphate-buffer containing M/100 $MgSO_4$ and then the addition of water to total volume. Test substances and activators when present were included in the following concentrations per ml of incubation medium: cysteine hydrochloride, 450 μg; adenosine-5-phosphate, 50 μg; pyridoxal phosphate, 15 μg; *alpha*-ketoglutarate, 1 mg; biotin, 10 mμg; alanine, 0.5 mg; glutamic acid, 0.5 mg; semicarbazide hydrochloride, 1 mg; pyruvate, 1 mg; phosphoglyceric acid, 1 mg; and serine, 0.5 mg. Activators were carefully neutralized where necessary before addition in order to control final pH. Except as otherwise indicated all experiments were conducted at pH 7.1 since this was the pH of optimum activity in the presence of all of the activators. This is in contrast to the previous observations of the desulfhydrase of *E. coli* (Desnuelle and Fromageot, 1939) where optimum activity was seen to be at pH 6.4; and, indeed, when well-washed intact cells were employed without activators, a broad range of activity was seen centering about pH 6.4. Further analysis of these observations is in progress, with the one fact thus far prominent that cell-free disintegrates prepared by sonic vibration in the presence of biotin, adenylic acid, and pyridoxal phosphate show best activity at pH values slightly above neutrality.

RESULTS AND DISCUSSION

The effects of the addition of various test substances upon the desulfhydrase system of *E. coli* as measured by hydrogen sulfide determination are tabulated in table 1.

In addition to the previously observed stimulation of pyridoxal phosphate in *Proteus* (Kallio, 1951), as well as with this organism in the present study, it is to be noted that adenosine-5-phosphate and biotin, alone and in combination with other activators, are stimulatory. These observations have been repeated a number of times, and although the exact significance insofar as site of action awaits

purification of the enzyme or enzymes involved, the marked similarity to serine and threonine deaminase is apparent. That the system is not identical to the dehydrase system (Wood and Gunsalus, 1949) is further supported by the observed finding that cysteine-adapted cells are relatively inactive on serine (deamination) and vice versa.

As concerns the function of pyridoxal phosphate, the theory entertained by us during the progress of the earlier observations, that it indicated a linked transamination (further implied by the stimulatory action of *alpha*-ketoglutarate) was considered untenable because of the failure of alanine-glutamic transaminase (Green *et al.*, 1945) to stimulate the system. If a linked transaminase was involved, the linkage could be assumed to occur through the formation of alanine by the reduction of aminoacrylic acid or iminopropionic acid. Alanine formed in such a manner could be assumed to be reactive with an active transaminase superimposed on the microbial system and a stimulation observed. Any stimulation observed by the addition of the pig heart transaminase could also be observed with boiled preparations indicating that it was probably due to the presence of liberated pyridoxal phosphate. It might be considered as a possibility, however, that the alanine formed is retained in such a manner as to be unavailable to added pig heart transaminase. We consider this possibility unlikely, however, because of our findings through another approach. If transamination was directly concerned, a subsequent deamination would be required to produce the observed amounts of ammonia. Experimentally we were unable to influence the equimolecular ratio of ammonia and hydrogen sulfide by the addition of the known amino acceptor *alpha*-ketoglutarate. Carefully standardized analytical techniques for ammonia by nesslerization and hydrogen sulfide by the previously described method showed an equimolar ratio under all conditions, and is in general agreement with the earlier finding of Desnuelle and Fromageot (1939).

As concerns the effects of the various inhibitors, it would seem that the marked inhibition of semicarbazide can be satisfactorily explained by its ability to combine with the active pyridoxal derivatives; that the phosphoglyceric acid and serine inhibitions can be most simply satisfied by assuming union of the desulfhydrase surface with these configurationally similar molecules, and the effect of alanine is seen as an indirect one resulting in increased amination of the active pyridoxal phosphate to the presumably inactive amine form. This view is strengthened by the observed stimulation (protection) by *alpha*-ketoglutarate and a frequently observed inhibition by aspartic and glutamic acids. In conjunction with this latter explanation it can be pointed out that Desnuelle *et al.* (1940) noted inhibition with many amino acids in the desulfhydrase of *Propionibacterium pentosaceum*.

That cysteine desulfhydrase and enolase are identical is unlikely since cells unadapted to cysteine are unaffected in their glycolytic ability.

SUMMARY

Cell-free disintegrates of a strain of *Escherichia coli* obtained by sonic vibration are capable of active desulfhydration of cysteine. The production of hydrogen sulfide is stimulated by adenosine-5-phosphate, pyridoxal phosphate, biotin, and

alpha-ketoglutarate and inhibited by phosphoglyceric acid, serine, alanine, and semicarbazide. A new method is described for the rapid and convenient estimation of hydrogen sulfide.

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