THE METABOLISM OF CYSTINE AND CYSTEINE BY PROTEUS VULGARIS AND PROTEUS MORGANII[1]

R. E. KALLIO AND J. R. PORTER

Department of Bacteriology, College of Medicine, The State University of Iowa, Iowa City, Iowa

Received for publication July 31, 1950

The microbial degradation of cystine and cysteine was first quantitatively investigated by Tarr (1933), who concluded after using *Proteus vulgaris* that cystine is reduced to cysteine, the latter then being anaerobically dissimilated to acetate, formate, hydrogen sulfide, and ammonia. Desnuelle and Fromageot in a series of studies (Desnuelle and Fromageot, 1939; Desnuelle, 1939; Desnuelle *et al.*, 1940) observed a system in *Escherichia coli* and *Proprionibacterium pentosaceum* that liberated hydrogen sulfide and ammonia from cysteine. The system in *E. coli* was partially adaptive, whereas in *P. pentosaceum* the enzyme was found to be constitutive in character. For each mole of cysteine utilized by these organisms one of ammonia and one of hydrogen sulfide were produced. Pyruvate accumulated in the presence of carbonyl reagent, but little quantitative data on this aspect of the problem was presented. Smythe (1941) studied a system in mammalian liver that attacked cysteine with the production of hydrogen sulfide, ammonia, and pyruvate. This system was called cysteine desulfhydrase (Smythe, 1944) because of its ability to liberate H_2S from cysteine. Binkley (1943) obtained the system in a cell-free state from *E. coli* and suggested that enolase, in addition to converting 2-phosphoglyceric acid to phosphopyruvate, also catalyzes the deamination of cysteine and serine inasmuch as his enolase preparations produced pyruvate from all three substrates. In this connection it is of interest to note that when Wood and Gunsalus (1949) purified serine and threonine dehydrase, it did not attack cysteine. This study was therefore undertaken to appraise cysteine desulfhydrase in bacteria and to study some of the characteristics of the system.

MATERIALS AND METHODS

Several strains of *Proteus vulgaris* and *Proteus morganii* were tested at the beginning of this study for their ability to produce hydrogen sulfide from the sulfur-containing amino acids cystine and cysteine. Since little difference was noted among the strains, under the conditions of these experiments, one representative of each species was chosen for further study. The strain of *Proteus vulgaris* used was the stock culture of this laboratory and the *Proteus morganii* was originally isolated by Dr. K. F. Rauss in Egypt (Rauss, 1936). The organisms were grown anaerobically in large flasks or pyrex bottles containing a medium consisting of 6 parts nutrient broth and 3 parts of fresh meat infusion broth ad-

[1] This study was supported in part by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, and the Central Scientific Fund of the College of Medicine, State University of Iowa.

justed to pH 7.4. Immediately prior to inoculation, sterile cysteine or cystine was added to this medium to make a final concentration of 0.05 per cent or 0.02 per cent, respectively. The inoculum was prepared by washing the entire growth from a 12-hour meat infusion agar slant.

Incubation was at 37 C and anaerobiosis was maintained by bubbling a vigorous stream of sterile, oxygen-free nitrogen through the incubating flasks. In addition to maintaining anaerobic conditions in the flasks the stream of nitrogen carried away the excess hydrogen sulfide, which was absorbed in a trap containing a strong cadmium acetate solution. The cells were harvested by Sharples centrifugation after 10 to 15 hours, since it was found that maximal desulfhydrase activity occurred at this time. Despite the heavy inoculum and the rich nature of the growth medium the yield of cells was relatively slight. This was apparently due to the dissolved hydrogen sulfide, which is known to poison certain enzymes and to precipitate various essential trace metals. The harvested cells were washed twice with 0.1 M phosphate buffer (pH 7.4) and then used immediately in metabolic studies. In certain experiments the organisms were rendered nonviable with toluene. When toluene treatment was employed, the time of contact between the washed cells suspended in phosphate buffer and redistilled toluene was 10 minutes at 25 C. Subcultures were made of all toluene-treated cells and these uniformly showed the organisms to be nonviable as indicated by the absence of growth. Cysteine- or cystine-adapted cells could also be obtained by growing the organisms aerobically, harvesting, and suspending the cellular mass in phosphate buffer to which had been added 0.05 per cent cysteine. The flask containing the mixture was placed in a water bath at 37 C, and sterile nitrogen was bubbled through it to maintain anaerobic conditions. The degree of adaptation of the cells was determined by measuring the amount of hydrogen sulfide produced after different periods of time. Adaptation usually reached a maximum in about 30 minutes, following which the cells were reharvested and washed twice with phosphate buffer.

Conventional Warburg equipment was used in the metabolic studies, and all experiments were conducted in triplicate under anaerobic conditions. In most cases incubation was for a period of 1 hour at 37 C. Controls were set up as follows for each series of experimental vessels: (1) one control was the same as the experimental flasks but lacked substrate to determine endogenous H_2S production; (2) another was identical to the experimental flasks but with the enzyme system boiled for 10 minutes—this control was a check on nonenzymatic H_2S production; and (3) a final control was provided in a vessel identical to the experimental flask but stopped after equilibration and analyzed for cysteine. Since the cysteine solution was made up and neutralized prior to each run, the last control established the amount of cysteine at the start of each experiment. There was little endogenous or nonenzymatic H_2S produced, but it seemed advisable to maintain such controls in light of the many nonenzymatic reactions that cysteine readily undergoes.

To determine hydrogen sulfide, 0.3 ml of approximately 1 N cadmium acetate solution were placed in the center well of each Warburg vessel. This solution

absorbed the free hydrogen sulfide and was converted to the insoluble sulfide of cadmium. After each experiment had proceeded the requisite time, the reaction was terminated by tipping in acid from a side bulb, and shaking was continued for an additional 10 minutes to insure complete absorption of hydrogen sulfide.

Quantitative estimation of hydrogen sulfide was made on the cadmium sulfide by acidifying with 6 N HCl to produce hydrogen sulfide. Immediately after acidification a known excess of 0.002 N iodine was added and the excess titrated with freshly prepared thiosulfate. Cystine and cysteine were determined by the phosphotungstic acid method of Shinohara and Padis (1936*a*,*b*). In a few in-

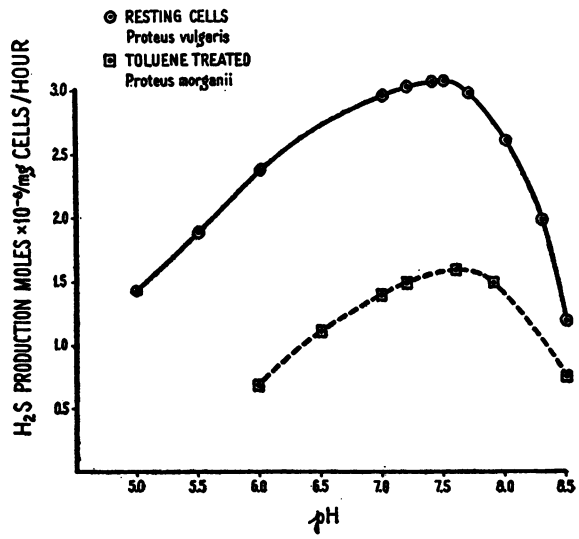

Figure 1. The effect of pH on cysteine desulfhydrase activity. Each flask contained 5 mg cells, 0.02 M cysteine, and buffer to make a final volume of 2.8 ml. Incubation period 1 hour at 37 C under anaerobic conditions.

stances elemental sulfur was determined by the micromethod of Guthrie (1938) using Warburg flasks and determining hydrogen sulfide as outlined above. Pyruvic acid determinations were made by the 2,4-dinitrophenylhydrazine method of Friedmann and Haugen (1943), using the "indirect" procedure and reading colors in a Coleman model 14 spectrophotometer.

RESULTS AND DISCUSSION

The influence of pH on bacterial cysteine desulfhydrase is shown in figure 1, in which it will be seen that the optimal pH for the reaction was 7.2 to 7.5 for resting cells of *Proteus vulgaris* and *Proteus morganii*, and slightly higher for toluene-killed cells of *Proteus morganii*. These values are in contrast to that (pH 6.4) quoted by Desnuelle and Fromageot (1939) for the hydrogen-sulfide-producing system in *Escherichia coli*. In all cases hydrogen sulfide was produced by resting cells from both cysteine and cystine, and by nonviable cells from cys-

teine, in the absence of external hydrogen donors—a condition claimed necessary for H_2S production by other workers. Our data do not, however, exclude the intervention of unknown endogenous hydrogen donors. When cystine was present as a neutral suspension, an identical pH curve was obtained with resting cells, whereas toluene-killed cells produced no H_2S from cystine.

The similarity of pH optima has no bearing on the question of whether or not cysteine is in the breakdown sequence of cystine. To determine this point the concept of simultaneous adaptation of Stanier was applied. Since Anderson (1947) questioned the adaptive nature of the system that attacks cysteine, it seemed important to reinvestigate this point and determine the cysteine desulfhydrase activities of cells grown under various conditions. The results of these experiments are shown in table 1. It is readily apparent that activity is enhanced by anaerobic growth provided cystine or cysteine is included in the growth medium. Although the system is not completely adaptive, Stanier's technique seems

TABLE 1

Cysteine desulfhydrase activity of Proteus vulgaris cells grown under various conditions*

GROWTH CONDITIONS	H_2S PRODUCTION MOLES $\times 10^{-6}$/ mg/hr
Agar surface (aerobic)	0.13
Agar surface (anaerobic)	1.03
Nutrient broth (aerated)	0.80
Nutrient broth (anaerobic)	1.45
Nutrient broth + 0.05% cysteine (anaerobic)	2.20
Nutrient broth + 0.10% cysteine (anaerobic)	2.00
Nutrient broth + 0.05% cystine (anaerobic)	2.22

* Determined by H_2S production in Warburg vessels. Each vessel contained 8 to 10 mg cells, 0.02 M cysteine, 0.05 M phosphate buffer pH 7.4; center well contained cadmium acetate. Incubation period 1 hour at 37 C under anaerobic conditions.

applicable in view of the increase in activity when cells are grown in contact with the specific substrate. Ajl (1950) has recently applied this modification of simultaneous adaptation to enzymes normally considered constitutive but whose quantitative aspects may be modified by appropriate growth conditions.

The results of cysteine and cystine metabolism by cells grown in the presence of these substrates are presented in figures 2 and 3. Inspection of these curves shows that though cystine-grown cells metabolize both amino acids without delay, cysteine-grown cells utilize cystine only following a lag period of about 30 minutes. Evidently cystine is first broken down to cysteine, and then each molecule of the latter undergoes subsequent metabolism.

The effects of various inhibitors on cysteine desulfhydrase in resting cells and toluene-killed cells are listed in table 2. It is of some interest that toluene treatment of *P. vulgaris* consistently yields completely inactive preparations, whereas similar treatment of *P. morganii* cells yields nonviable cells that are active in producing hydrogen sulfide from cysteine but not from cystine. Ordinarily *P. morganii* does not produce H_2S on the common diagnostic media (e.g., Kligler's

iron agar) but under the conditions used in this study it metabolizes cystine and cysteine at a rate very similar to that of *P. vulgaris*. Noteworthy in the

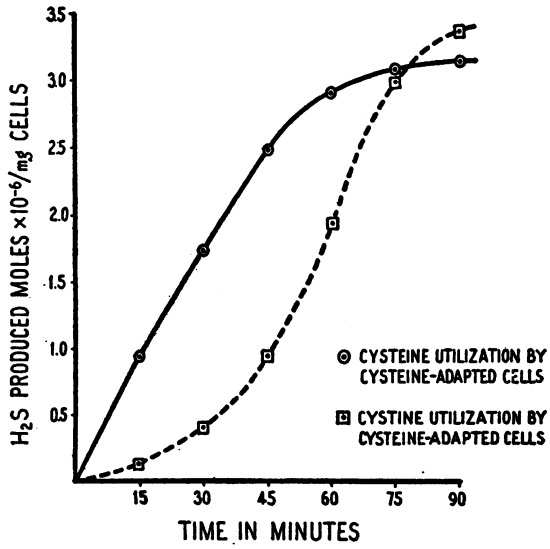

Figure 2. Utilization of cysteine and cystine by *Proteus vulgaris* cells grown in cysteine-containing medium. Conditions as in figure 1.

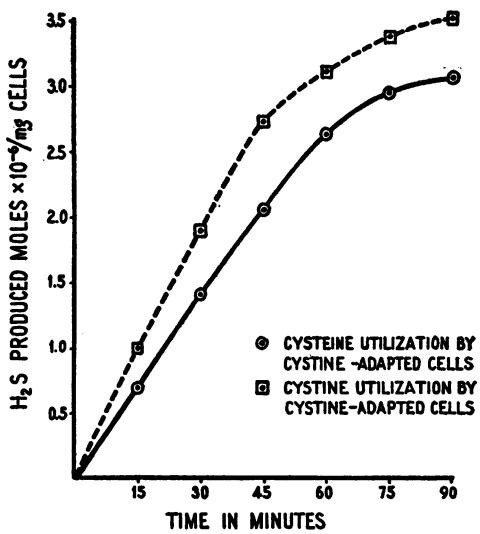

Figure 3. Utilization of cysteine and cystine by *Proteus vulgaris* cells grown in cystine-containing medium. Conditions as in figure 1.

inhibition studies is the lack of retardment by reagents capable of forming complexes with divalent metals, especially in view of the claim that divalent metals are necessary for the system (Binkley, 1943). The importance of functional

carbonyl groups is evident from the powerful inhibition by carbonyl reagents. No inhibition was observed in the presence of amino acids even though the concentrations were raised to equal that of the cysteine. This observation is in agreement with the study of Lawrence and Smythe (1943) but it conflicts with the results of Desnuelle and Fromageot (1939). Sodium azide even in relatively

TABLE 2

*Effects of various inhibitors on cysteine desulfhydrase based on H_2S production**

INHIBITOR	CONCENTRATION	PER CENT INHIBITION	
		Resting cells *P. vulgaris*	Toluene-killed *P. morganii*
Sodium arsenite	0.005 M	100	100
Sodium arsenite	0.0005 M	90	96
Iodoacetic acid	0.001 M	75	—
Serine	0.015 M	10	10
Serine	0.020 M	10	19
Alanine	0.021 M	9	16
Hydroxylamine	0.001 M	90	89
Phenylhydrazine	0.001 M	—	88
Semicarbazide	0.001 M	65	75
α-α'Dipyridyl	0.005 M	—	10
8-Hydroxyquinoline	0.001 M	—	0

* Conditions as in table 1 except for the addition of the inhibitors.

TABLE 3

*Sodium azide inhibition of anaerobic pyruvate utilization by Proteus vulgaris**

AZIDE ADDITIONS	PYRUVATE UTILIZED (PYRUVATE ADDED—PYRUVATE RECOVERED) MOLES $\times 10^{-6}$/mg/hr
No azide	4.5
0.001 M azide	4.6
0.004 M azide	4.2
0.008 M azide	2.6
0.010 M azide	1.3
0.015 M azide	0.9

* Each experimental vessel contained 60 moles $\times 10^{-6}$ pyruvate, 8 mg cells, azide as noted, and phosphate buffer pH 7.4 to a total volume of 2.8 ml. Incubation period 1 hour at 37 C under anaerobic conditions.

high concentrations does not appreciably inhibit the production of hydrogen sulfide from cysteine, but it does inhibit markedly the ability of the cells to utilize pyruvate. This fact is shown in table 3.

Following the incubation of resting cells of *P. vulgaris* or *P. morganii* in the presence of 0.015 M azide, pyruvate was isolated as the 2,4-dinitrophenylhydrazone. A purified sample of this derivative melted at 214 C (uncorrected), comparing very favorably with a similar derivative prepared from purified lithium

pyruvate that also melted at 214 C (uncorrected). In the light of the azide action on pyruvate, experiments were conducted using azide-inhibited cells. After incubation, analyses were made for cysteine, pyruvate, ammonia, and hydrogen sulfide. Typical results of one of the experiments are shown in table 4. The values for hydrogen sulfide are probably minimal inasmuch as known amounts of hydrogen sulfide added as sodium sulfide and subsequently acidified could be recovered only to the extent of 87 to 90 per cent. Although ammonia values were erratic, they were never such that we could conclude that no ammonia is produced. This fact assumes some interest in view of a recent report in which a mammalian liver enzyme was found to be capable of producing hydrogen sulfide but not ammonia from cysteine (Binkley and Okeson, 1950). Values for pyruvate are in excellent accord with the concept that for each mole of cysteine

TABLE 4

Anaerobic cysteine dissimilation by azide-inhibited resting cells of Proteus vulgaris

CYSTEINE DISAPPEARING	H_2S PRODUCED	NH_3 PRODUCED	PYRUVATE PRODUCED	S PRODUCED
	moles $\times 10^{-6}$*/mg/hr*			
2.4	1.8	2.0	2.0	—
2.0	1.3	0.6	2.5	—
2.2	1.8	0.8	2.4	—
2.1	1.6	1.0	1.6	0.0
3.0	1.9	1.0	1.7	0.0
2.6	2.1	—	2.8	0.1
2.5	1.6	1.1	2.0	0.1
2.0	1.5	2.1	2.3	—
Averages				
2.4	1.8	1.2	2.1	0.05

Conditions same as in table 1 except 0.015 M azide added.

disappearing, one of pyruvate is produced. Elemental sulfur values were zero or so low that they appear to be of little significance.

Analyses performed in the same way following incubation of cysteine and nonviable toluene-treated cells of *P. morganii* confirmed the finding that cysteine is anaerobically dissimilated to hydrogen sulfide, ammonia, and pyruvate. The toluene preparations, however, were completely inactive against pyruvate and cystine. In this connection it is of interest that some hydrogen sulfide was produced from homocysteine by *P. morganii* in the resting state or in toluene-killed preparations, whereas *P. vulgaris* was inactive toward homocysteine under the same conditions.

The cysteine desulfhydrase activity of toluene-treated *P. morganii* cells could be reduced considerably by holding ("aging") at pH 4.5 in phosphate buffer for 1 to 2 hours. This activity could then be restored by the addition of boiled *P. morganii* cell extracts, but not by any of the known vitamins or by Mg^{++}, Mn^{++}, Co^{++}, Zn^{++}, or various combinations of vitamins and metals. In view of the

suggested identity of serine dehydrase and cysteine desulfhydrase the activators found to be necessary for serine dehydrase activity by Wood and Gunsalus (1949) were used in an attempt to restore the activity of the aged preparations. The results in table 5 indicate that neither glutathione plus adenosine-3-phosphate nor glutathione plus adenosine-5-phosphate are operative in this system. The addition of magnesium to these combinations likewise does not cause reactivation. These results confirm, in a negative way, the finding of Wood and Gunsalus that serine dehydrase and cysteine desulfhydrase are not the same enzyme.

Studies on cell-free preparations of cysteine desulfhydrase are being conducted and will be reported in a subsequent paper.

TABLE 5

Reactivation of "aged," toluene-killed Proteus morganii cells toward cysteine

EXPERIMENTAL CONDITIONS	H_2S PRODUCED MOLES $\times 10^{-4}$/HOUR
Toluene-treated cells	4.2
Treated cells aged 1 hour at pH 4.5	1.6
Treated cells aged 2 hours at pH 4.5	1.0
Aged cells + Mg^{++}	1.5
Aged cells + Mg^{++} + biotin	1.4
Aged cells + Mg^{++} + adenosine-3-PO_4	1.2
Aged cells + adenosine-3-PO_4 + glutathione + Mg^{++}	1.4
Aged cells + adenosine-5-PO_4 + glutathione + Mg^{++}	1.4
Aged cells + boiled cell extract	3.9

SUMMARY

Proteus vulgaris and *Proteus morganii* anaerobically dissimilate cystine to cysteine, which in turn yields pyruvate, ammonia, and hydrogen sulfide. Cysteine desulfhydrase does not appear to be identical with serine and threonine dehydrases.

REFERENCES

AJL, S. J. 1950 Acetic acid oxidation by *Escherichia coli* and *Aerobacter aerogenes*. J. Bact., **59**, 499–507.

ANDERSON, K. E. 1947 Some studies on amino acid metabolism by the genus *Proteus*. Science Studies St. Bonaventure Coll., **13**, 2–20.

BINKLEY, F. 1943 On the nature of serine dehydrase and cysteine desulfurase. J. Biol. Chem., **150**, 261–262.

BINKLEY, F., AND OKESON, D. 1950 Purification of the enzyme responsible for the cleavage of cystathione. J. Biol. Chem., **182**, 272–277.

DESNUELLE, P. 1939 La decomposition anaérobie de la cysteine par *E. coli*. II. Son role dans la degradation de la cystine. Enzymologia, **6**, 387–391.

DESNUELLE, P., AND FROMAGEOT, C. 1939 La decomposition anaérobie de la cysteine par *E. coli*. I. Existence d'une cysteinase, ferment d'adaptation. Enzymologia, **6**, 80–87.

DESNUELLE, P., WOOKY, E., AND FROMAGEOT, C. 1940 Sur la degradation anaérobie de la cysteine et de la cystine par *Propionibacterium pentosaceum*. Enzymologia, **8**, 225–240.

FRIEDMANN, T. E., AND HAUGEN, G. E. 1943 Pyruvic acid. II. The determination of ketoacids in blood and urine. J. Biol. Chem., **147**, 415–441.

GUTHRIE, J. D. 1938 A new method for the determination of glutathione in tissues based on its reaction with sulfur to form hydrogen sulfide. Contrib. Boyce Thompson Inst., **9**, 223–232.

LAWRENCE, J. M., AND SMYTHE, C. V. 1943 The partial purification and some of the properties of the enzyme in rat liver that forms hydrogen sulfide from cysteine. Arch. Biochem., **2**, 225–234.

RAUSS, K. F. 1936 The systematic position of Morgan's bacillus. J. Path. Bact., **42**, 183–192.

SHINOHARA, K., AND PADIS, K. E. 1936*a* The determination of thiol and disulfide compounds, with specific reference to cysteine and cystine. J. Biol. Chem., **112**, 697–708.

SHINOHARA, K., AND PADIS, K. E. 1936*b* Application of the modified phospho-18-tungstic acid method for the determination of cysteine, cystine and ascorbic acid in urine. J. Biol. Chem., **112**, 709–721.

SMYTHE, C. V. 1941 The utilization of cysteine by rat liver with the production of hydrogen sulfide. J. Biol. Chem., **142**, 387–400.

SMYTHE, C. V. 1944 Some enzyme reactions of sulfur compounds and their possible interrelationship. Ann. N. Y. Acad. Sci., **45**, 425–435.

TARR, H. L. A. 1933 The anaerobic decomposition of L-cystine by washed cells of *Proteus vulgaris*. Biochem. J., **27**, 759–763.

WOOD, W. A., AND GUNSALUS, I. C. 1949 Serine and threonine deaminases of *Escherichia coli:* activators for a cell-free enzyme. J. Biol. Chem., **181**, 171–182.