THE IRREVERSIBILITY OF METHIONINE SYNTHESIS FROM CYSTEINE IN PASTEURELLA PESTIS[1]

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In a study of the nutrition of *Pasteurella pestis*, strain A1122, it was found that this organism requires both thiosulfate and methionine for growth. In a similar investigation of both virulent and avirulent strains of *P. pestis*, Hills and Spurr (1950) and Rockenmacher (1950) made the observation that both cystine and methionine are essential. This natural requirement for two sulfur-containing compounds by several strains of *P. pestis* and the indication that the cysteine-to-methionine series of reactions (Horowitz, 1947) would not be complicated by the presence of an alternate pathway for the transfer of the sulfur of methionine to cysteine, as seems to be the case in mutants of *Neurospora* (Horowitz, 1947) and *Escherichia coli*, strain 15 (Lampen *et al.*, 1947), prompted a more detailed exploration of the sulfur metabolism of this organism.

MATERIALS AND METHODS

Pasteurella pestis, strain A1122, avirulent, originally isolated from a virulent strain by Jawetz and Meyer (1943) was employed in this study.

Stock cultures were maintained originally on blood hormone agar and subsequently on a casein hydrolysate glucose medium (Englesberg *et al.*, 1951). The latter medium was also employed in growing this organism for nutritional investigations. For this purpose a portion of the growth of a 24-hour, 30 C slant culture was scraped off and emulsified in M/20 phosphate buffer, pH 7, centrifuged in the cold, and resuspended in buffer. In early experiments this washing procedure was repeated twice, but subsequently only one washing was found necessary. The washed suspension was diluted with buffer so that in experiments employing liquid media the final concentration of cells per ml was about 1×10^5. In using the auxanographic technique, a final suspension of about 5×10^7 cells per ml of agar medium was employed.

In determining the nutritional requirements of this organism, the auxanographic technique and streaked plates were employed for the most part. Liquid media were avoided since growth therein in many cases resulted from mutation and selection. Liquid media were used only to verify results obtained with agar media and when traces of sulfide found in agar made the use of agar difficult.

Growth in liquid media was determined by inoculating *P. pestis* into 2.5 or 3 ml of medium in duplicate test tubes (16 × 150 mm). The tubes were incubated upright or on their sides at 30 C for 72 hours. The cultures were then diluted twofold with saline or phosphate buffer, and growth was measured as the per cent light transmission in a Coleman spectrophotometer at 650 mμ.

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The basal medium used had the following per cent composition: glucose 0.2, NH_4Cl 0.1, $CaCl_2$ 10^{-3}, $FeCl_3(6H_2O)$ 2.5×10^{-4}, $MgCl_2(6H_2O)$ 0.04, and agar (when employed) 1.5. KH_2PO_4 and Na_2HPO_4, pH 7, were added to yield a M/20 solution. The media were prepared by autoclaving together agar and distilled water in the case of solid media, or phosphate and distilled water for liquid media, and adding each of the other components separately. The preparations were conducted in this way to avoid precipitation of the salts and to prevent inhibition of the growth of *P. pestis* which occurred when phosphate and agar were autoclaved together (Englesberg *et al.*, 1951). Glucose was sterilized by filtering through sintered glass, and the remaining chemicals were sterilized by autoclaving. Amino acids and inorganic sulfur compounds were added when desired. The sulfur-containing amino acids and all inorganic sulfur compounds except $MgSO_4$ were sterilized through sintered glass. $MgSO_4$ and the remaining amino acids were sterilized by autoclaving.

All chemicals employed in this investigation were commercial products except the L-cystathionine, which was generously supplied by Dr. N. H. Horowitz.

RESULTS

Synthetic medium for strain A1122: P. pestis grew in liquid and solid media upon the addition of 0.4 mg of each of the following compounds per liter of basal medium: DL-phenylalanine, DL-valine, DL-isoleucine, DL-methionine, and sodium thiosulfate ($5H_2O$). The omission of any one or combination of these compounds prevented growth. Doudoroff (1943) reported growth of this strain of *P. pestis* in a glucose mineral medium supplemented with just phenylalanine and cystine under conditions in which "selection probably occurred" (large inocula, slow growth followed by rapid growth on subculture). That mutation and selection had occurred in this case was substantiated with the isolation by us of mutants requiring just phenylalanine and cystine.

Sulfur requirements of strain A1122: Table 1 illustrates in detail the sulfur requirements of this strain. No single sulfur-containing amino acid or inorganic compound can satisfactorily replace the thiosulfate and methionine required by this organism. In the presence of thiosulfate only, DL-homocysteine[2] or L-cystathionine completely replaces methionine, whereas sulfate, sulfite, sulfide, and L-cysteine[2] are inactive. In the presence of DL-methionine, only sulfite, sulfide, or L-cysteine replaces thiosulfate, while sulfate or DL-homocysteine failed to yield any growth at all and L-cystathionine yielded but scant growth.

Isolation of a thiosulfate-requiring methionine-independent mutant (M1): When the basal agar medium containing DL-phenylalanine, DL-valine, DL-isoleucine, and thiosulfate was streaked heavily with the wild type, faint growth in 96 hours was followed by the appearance of secondary colonies. Several such mutant colonies were isolated in pure culture. All have the same characteristic sulfur requirements: Each grew with sulfite, thiosulfate, sulfide, or L-cysteine,

[2] Since *Pasteurella pestis*, strain A1122, wild type and mutant M1 can use DL-homocystine interchangeably with DL-homocysteine, and L-cystine with L-cysteine, reference shall be made only to the reduced forms of these compounds.

TABLE 1

Growth of Pasteurella pestis wild type and mutant M1 on various sulfur sources

The organisms were inoculated into tubes (see Materials and Methods) containing the basal medium plus 0.4 mg/L of each of the following: DL-phenylalanine, DL-valine, and and DL-isoleucine. The sulfur-containing compounds were added as indicated, yielding a final concentration of 10 μg/ml. When growth did not occur, the effect of varying the concentrations of the particular S compounds from 5 to 100 μg/ml was tested. Unless otherwise indicated this had no detectable effect on growth.

S SOURCES ADDED	AMOUNT	PER CENT LIGHT TRANSMISSION*					
		No additional S added		$Na_2S_2O_3(5H_2O)$, 10 μg/ml		DL-Methionine, 10 μg/ml	
		Wild type	Mutant	Wild type	Mutant	Wild type	Mutant
	μg/ml						
$MgSO_4(7H_2O)$	10	0	0	0	+++	0	0
Na_2SO_3†	10	0	0	0	++++	+++	+++
	100	0	+−	0			
	250	0	+++				
$Na_2S_2O_3(5H_2O)$	10	0	+++			+++	++++
$Na_2S(9H_2O)$	10	0	+++	0	+++	++++	++++
L-Cystine	10	0	+++	0	++++	++++	++++
L-Cysteine	10	0	++++	0	+++++	++++	++++
L-Cystathionine‡	5	0	0	+++	++++	0	0
	10	+−	+−				
	20	+−	+−			+	+−
	40	+	+				
DL-Homocystine	10	0	0	+−	++++	0	0
	40	0	0	+++		0	0
DL-Homocysteine	10	0	0	+−	++++	0	0
	40	0	0	++++		0	0
DL-Methionine	10	0	0	+++	++++		

* The per cent light transmission is represented as follows:

100 as 0	70–79 as +++
95–99 as +−	60–69 as ++++
90–94 as +	50–59 as +++++
80–89 as ++	

† Maximum growth of the mutant in sulfite media was delayed until 96 hours and did not occur at all with dilute solutions of this chemical. The failure to obtain significant growth with concentrations of less than 100 μg/ml and the delay in growth observed with the higher concentrations are probably the result of the rapid decomposition of sulfite in air and the inhibition of growth with the higher concentrations employed, respectively. This explanation seems likely since growth similar to that achieved with 250 μg of Na_2SO_3/ml under the conditions of this experiment was obtained by adding to the cultures a total of 80 μg of Na_2SO_3/ml in small amounts over a period of 96 hours. The homogeneity of the growth of M1 on solid medium containing 250 μg of sulfite per ml as the sole added S source (no growth occurred in similar medium in which Na_2SO_3 was omitted) indicates that the delay in growth observed in liquid media is not the result of further mutation.

‡ Because the supply of this compound was limited, 40 μg/ml was the highest concentration tested.

failed to grow with sulfate, DL-homocysteine, or DL-methionine as sole sulfur sources, and yielded but scant growth with L-cystathionine. (The slight growth of both the M1 mutant and the wild type which occurs in the presence of cystathionine is thought to be the result of a contaminating sulfur source.) In the presence of methionine, the mutants behaved as did the wild type (table 1).

DISCUSSION

Since methionine and cysteine are necessary components of most protein molecules, it appears that the dual sulfur requirement of *P. pestis* is essentially a requirement for these two compounds. Evidence presented indicates that the inability of this organism to synthesize either cysteine or methionine is the result of: (1) at least two breaks in the reaction chain leading from sulfate to methionine (see figure 1), one between sulfate and sulfite and the other between cysteine and cystathionine and (2) the lack of any alternate pathway for the transfer of the S of methionine to cysteine. The isolation directly from the wild

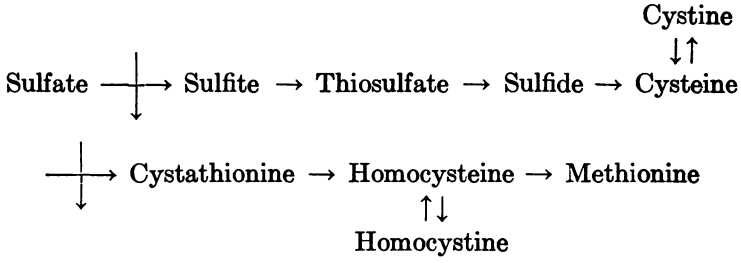

Figure 1. Pathway for methionine synthesis.

type of a "spontaneous" mutant able to use sulfite, thiosulfate, sulfide, or cysteine as sole sulfur source indicates that a gain mutation probably occurred at the cysteine-cystathionine site, enabling this mutant to synthesize methionine from the previously mentioned compounds. The fact that this mutant is still unable to use DL-methionine, DL-homocysteine, or L-cystathionine as sole sulfur source demonstrates the irreversibility of the cysteine-to-methionine conversion in this organism.

Similar evidence of the irreversibility of the cysteine-to-methionine synthesis was presented by Simmonds (1948), who showed that a cystineless strain of *E. coli*, strain K12, was unable to use methionine for cystine synthesis, although it was able to make methionine from cystine. Hift and Wallace (1949) demonstrated a similar phenomenon with several strains of lactic acid bacteria.

Thus *P. pestis* and the previously mentioned organisms, unlike the mammal, *Neurospora*, and *E. coli*, strain 15, are unable to convert methionine S into cysteine S. In the rat there is fairly good evidence that this conversion goes through homocysteine and cystathionine (Tarver and Schmidt, 1939; du Vigneaud *et al.*, 1942; du Vigneaud *et al.*, 1944). In *Neurospora* (Horowitz, 1947) and to a lesser extent in *E. coli*, strain 15 (Lampen *et al.*, 1947), however, although there is evidence for the conversion of cysteine to methionine, as

illustrated in figure 1, there is no evidence for or against the existence of the reverse reaction. In fact, the data suggest the existence of a pathway of conversion of methionine S to cysteine S which may be independent of cystathionine and homocysteine. In *Neurospora* this is indicated by the fact that the wild type can use sulfate or methionine as the sole source of sulfur (in the presence of a negligible amount of S present in biotin), and artificially produced single gene mutants, unable to use cysteine (strain me 3), cystathionine (strain me 2), or homocysteine (strain me 1) for growth and therefore for methionine synthesis, are all able to use methionine for cysteine synthesis. In *E. coli*, strain 15, somewhat similar data have been presented.

Horowitz (1950) reported that *Neurospora* can not use sulfide and postulated that the sulfur atom reacts with an organic compound before attaining the sulfide level of reduction. This is not so in *P. pestis* which is able to use sulfide and is therefore similar in this respect to *E. coli* (Lampen *et al.*, 1947).

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SUMMARY

Pasteurella pestis, strain A1122, requires as nitrogen and sulfur sources for growth DL-phenylalanine, DL-valine, DL-isoleucine, DL-methionine, and thiosulfate.

In the presence of DL-methionine, thiosulfate is replaceable only by either L-cysteine, sulfide, or sulfite, and in the presence of thiosulfate, DL-methionine is replaceable only by DL-homocysteine or L-cystathionine. This dual sulfur requirement is explained by the inability of this organism to synthesize cysteine and methionine from inorganic sulfate by virtue of breaks probably in the conversion of sulfate to sulfite and of cysteine to cystathionine and the lack of an alternate pathway for converting the sulfur of methionine to the S of cysteine.

A "spontaneous" mutant was isolated which utilizes sulfite, thiosulfate, sulfide, or L-cysteine as sole sulfur source, indicating that a gain mutation probably occurred at the cysteine-to-cystathionine site, thus enabling this mutant to synthesize methionine from any of the above four compounds. The fact that this mutant is still unable to use DL-methionine, DL-homocysteine, or L-cystathionine as sole sulfur source demonstrates the irreversibility of the cysteine-to-methionine synthesis in this organism.

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