The Assimilation of Amino Acids by Bacteria

17. SYNTHESIS OF GLUTATHIONE BY EXTRACTS OF *ESCHERICHIA COLI*

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Several authors have suggested that glutathione plays a part in the synthesis of proteins. Thus Roberts & Bolton (1952) have shown that when cells of *Escherichia coli* containing ^{35}S -labelled glutathione grow in the presence of unlabelled sulphate, approximately half of the ^{35}S of glutathione is incorporated into the proteins and half is released into the medium. Abelson (1952) reported that a very high proportion of cysteinylglycine residues occur in the proteins of *Esch. coli*. Gale & Folkes (1953*a*) have shown that there is an increase in protein content when washed cell suspensions of *Staphylococcus aureus* are incubated with a complete mixture of amino acids and glucose. If purines and pyrimidines are added to this mixture the nucleic acid content of the cells increases, and a marked acceleration in the rate of protein synthesis also occurs. Bactericidal concentrations of the antibiotics chloramphenicol, aureomycin, and terramycin inhibit protein synthesis in *Staph. aureus* (Gale & Folkes, 1953*b*). Whereas penicillin and bacitracin have no direct effect on the increase in cellular protein obtained when cells are incubated in the presence of a complete mixture of amino acids, the incorporation of labelled glutamic acid into cell protein which occurs when the cells are incubated with glucose and glutamic acid alone is inhibited by these two antibiotics (Gale & Folkes, 1953*c*) and consequently they may be assumed to interfere with some stage in protein metabolism. Hanes, Hird & Isherwood (1950) found that penicillin inhibits transpeptidation reactions involving glutathione in the presence of sheep-kidney suspensions, while Binkley & Olson (1951) showed that the breakdown of glutathione to glutamic acid and cysteinylglycine by this tissue is also inhibited, possibly competitively, by penicillin. It was thought that a study of the synthesis of glutathione by bacterial extracts and the sensitivity of this reaction to antibiotics might throw some light on the possible role of glutathione in protein synthesis. Since there is a structural relationship between glutathione and penicillin it was of particular interest to study the effect of this antibiotic on the synthesis of glutathione.

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thione. Johnston & Bloch (1951) have obtained a synthesis of glutathione by cell-free extracts of liver. The synthesis of glutathione by cell-free extracts of *Esch. coli* and the action of antibiotics thereon, are the subject of this paper.

METHODS

Organism. The organism used throughout these studies was *Escherichia coli* N.C.T.C. no. 86 obtained from the National Collection of Type Cultures, London.

Growth conditions. The organism was grown for 15 hr. at 37° in casein digest broth containing 2% (w/v) glucose and aerated with air or O_2 . The medium (1.5 l.) was inoculated with 15 ml. of an 8 hr. culture in casein digest broth. Yields of bacteria obtained under these conditions varied from 0.60 to 0.85 mg. dry wt./ml. For some preparations higher yields of bacteria were obtained by growth for 15 hr. at 37° in Roux bottles on the same medium solidified with 3% (w/v) agar.

Growth was determined turbidimetrically on a Hilger Spekker absorptiometer previously calibrated against known dry weights of the organism used.

Preparation of extracts. The organisms were harvested by centrifugation and washed once in cold distilled water containing 0.001 M-cysteine and 0.0001 M-potassium phosphate buffer at pH 7 ('extraction mixture') in a tared tube. Extracts were obtained from the wet cells either by grinding with 2.5 times their weight of powdered alumina in a pre-cooled mortar (McIlwain, 1948) or by crushing with an equal weight of alumina in the Hughes press (Hughes, 1951). In each case the mixture was treated with cold extraction mixture (5 ml./g. wet wt. of bacteria) cooled in ice, and centrifuged at 11 000 *g* on a Servall centrifuge for 5 min. The yellowish, slightly opalescent supernatant fluid was used directly, or after dialysis against cold extraction mixture for 4 hr. with stirring. Such extracts were stored at -18° and normally kept their activity for at least 4 months. Active extracts were also obtained by shaking a thick suspension of the organisms in extraction mixture at 25 keyc./sec. using a Mullard magnetostriktor oscillator or with glass beads on the Mickle machine (Mickle, 1948). In experiments where the activity of various extracts was compared, a portion of each extract was dried for 3 hr. at 105° and the results expressed per mg. dry weight of the sample.

Incubation mixtures and conditions. The standard medium for incubation was: 1.0 ml. extract; 0.5 ml. 0.5 M-potassium phosphate buffer, pH 7.7; 0.2 ml. 0.16 M-sodium glutamate, pH 7.4; 0.2 ml. 0.16 M-glycine; 0.1 ml. 0.16 M-cysteine, pH 7.4; 0.3 ml. 0.22 M-magnesium hexose diphosphate

(HDP); 0.1 ml. 0.006M-diphosphopyridine nucleotide (DPN); 0.1 ml. 0.06M-adenosine triphosphate (ATP); water to 3 ml. The pH of the mixture was between 7 and 7.4.

Incubations were carried out in test tubes (4 × 0.5 in.) in air at 37° for 1 or 1.5 hr. At zero time, a 1.0 ml. sample of the mixture was heated in a test tube in a bath of boiling water for 2 min. with shaking. After cooling, the precipitated protein was removed by centrifugation. The same procedure was adopted for the mixture after incubation. Glutathione was measured in the supernatants and the difference between the final and initial values taken as a measure of the net synthesis of glutathione by the extract.

Estimation of glutathione. Glutathione was measured by the glyoxalase method of Woodward (1935). The possibility was entertained that the relatively high concentrations of phosphate present in the incubation mixture might interfere with the assay of glutathione. It was shown, however, that the addition of similar concentrations of phosphate did not affect the results.

Chromatography. Samples of the incubation mixture were examined by paper chromatography for the presence of glutathione. Samples of the supernatant (0.5 ml.) obtained after deproteinization by heating were treated with twice their volume of warm ethanol containing approximately 0.01M-N-ethylmaleimide which facilitates the migration of glutathione and cysteine on the paper (Hanes *et al.* 1950). Any precipitates formed were removed by centrifugation and the supernatant solutions dried down in a vacuum desiccator. The product was dissolved in 0.1 ml. water and 2–4 μ l. spotted on Whatman paper no. 1 or no. 3. Descending chromatograms were developed by a solvent mixture consisting of 80% propanol:20% water (v/v). Papers were dried for 30 min. at 80° and the position of peptides and amino acids determined by spraying with 0.1% ninhydrin in chloroform and heating to 80–100° for 5 min.

RESULTS

Synthesis of glutathione by extracts of Escherichia coli

Early experiments to determine the components required for the synthesis of glutathione by bacterial extracts are shown in Table 1. In most experiments the increase of glutathione during the incubation amounted to 2–5 times that initially present in the extract. In a typical experiment the initial amount was 210 μ g. and after 90 min. this had increased to 660 μ g. indicating a net synthesis of 450 μ g. by 1 ml. of the extract.

Little synthesis occurred in the absence of glycine and/or glutamate, and none after heating the enzyme extract to 100° for 10 min. Bloch, Snoko & Yanari (1952) have shown that ATP is required for incorporation of the amino acids into glutathione by liver extracts. *Esch. coli* contains an active adenosine triphosphatase (ATP-ase) so that added ATP was rapidly destroyed. Good synthesis was obtained, however, if ATP was generated in the incubation mixture by glycolysis of added magnesium HDP in the presence of DPN and a small amount of ATP or adenosine diphosphate (ADP). If neither

ATP nor DPN was added to the incubation mixture synthesis was decreased by 50%, and there was little or no synthesis in the absence of both. When HDP was omitted synthesis did not occur even when magnesium ions were added; in the presence of sodium HDP no glutathione was synthesized unless magnesium ions were added. Under the conditions tested, only in isolated cases has it been possible to replace HDP, ATP and DPN by a higher concentration of ATP alone.

Table 1. *Requirements for glutathione synthesis by extracts of Escherichia coli*

(Complete incubation mixture contains: 1.0 ml. extract; 0.5 ml. 0.5M-potassium phosphate buffer, pH 7.4; 0.2 ml. 0.16M-sodium glutamate, pH 7.4; 0.2 ml. 0.16M-glycine; 0.1 ml. 0.16M-cysteine, pH 7.4; 0.3 ml. 0.22M-Mg HDP; 0.1 ml. 0.006M-DPN; 0.1 ml. 0.06M-ATP and water to 3.0 ml. Incubations were carried out for 90 min. at 37° and results expressed as the amount of glutathione produced in the 3.0 ml. incubation.)

Incubation mixture	Glutathione synthesized (μ g.)
Complete system	300
Omit amino acids	6
Omit glycine	30
Omit glutamate	24
Omit DPN	150
Omit ATP	168
Omit ATP and DPN	18
Omit HDP	0
Extract heated to 100°	0

Chromatographic examination of the products after the extract was incubated with added glutathione, showed that no breakdown of glutathione occurred, and consequently this system was particularly suitable for the study of glutathione synthesis.

Effect of time of growth on glutathione content of Escherichia coli

Determinations of the amounts of glutathione in bacteria at different stages in the growth cycle indicated that the cells contain more at the beginning of active growth (5–6 hr.) than when grown for longer periods. Kopper (1951) also found that 3–5 hr. cultures of *Esch. coli* reduced nitrate and methylene blue more readily than did older cells; activities which might be associated with the thiol content of the cells. Initial attempts to obtain an extract capable of synthesizing glutathione were therefore carried out using cultures grown for 5–6 hr. However, it was found that the most active extracts were obtained from cells harvested at the end of their period of active growth (Table 2) and in subsequent experiments 15 hr. cultures were used for the preparation of extracts.

Table 2. *Effect of age of culture on the synthesis of glutathione by extracts of Escherichia coli*

Age (hr.)	Growth (mg. dry wt./ml.)	Activity/mg. dry wt. of extract*
Expt. 1		
3.75	0.24	11.3
6	0.61	13.8
9.5	0.89	18.6
Expt. 2		
4.25	0.44	5.85
8.25	0.72	8.1
13	0.72	11.9

* The activity is expressed as $\mu\text{g.}$ of glutathione synthesized/mg. dry wt. of enzyme extract/hr., under the conditions described in the section on methods.

Rate of synthesis by cell-free extracts

Fig. 1 shows a typical progress curve of the reaction studied at pH 7.3 and 37°. The glutathione synthesis was linear for the first 90 min. and the rate

Fig. 1. Progressive synthesis of glutathione from its component amino acids by extracts of *Esch. coli*. Experimental details in text.

then decreased with time, although the reaction continued very slowly up to and over 3 hr. As yet no attempt has been made to determine why the rate of reaction decreases with time. It may be due to oxidation of cysteine, change in pH, lack of high-energy phosphate, or a combination of these factors.

Fig. 2 shows that the amount of glutathione formed is proportional, within experimental error, to the amount of extract present.

Identification of the product

Comparison of chromatograms of the reaction mixture before and after incubation (4 hr.) demonstrates an increase of material running in a similar position to glutathione. Using 75 ml. of extract and

corresponding amounts of the other components of the incubation mixture, an attempt was made to obtain larger quantities of the material for chemical identification. The product was precipitated first as the cadmium derivative and then as the copper mercaptide (Waelsch & Rittenberg, 1941). After removal of the copper the material proved a potent activator of glyoxalase and its chromatographic behaviour was identical with glutathione. On hydrolysis the substance gave glutamic acid, glycine, and cysteine acid presumably derived from cysteine.

Effect of antibiotics

The effect of a series of antibiotics on glutathione synthesis by *Esch. coli* extracts was studied. Penicillin, bacitracin, dihydrostreptomycin, streptomycin, polymyxin, neomycin, terramycin, aureomycin and chloramphenicol at concentrations of 100 $\mu\text{g.}/\text{ml.}$ had no significant effect on the reaction. In view of the fact that penicillin reacts with cysteine (Cavallito, 1946) and might be inactivated, the experiment was repeated using concentrations of penicillin up to 1 mg./ml. No inhibition was observed.

Fig. 2. Effect of enzyme concentration on glutathione synthesis. Incubations carried out for 90 min. at 37°, under conditions described in Methods section.

DISCUSSION

The system extracted from *Esch. coli* which synthesizes glutathione from its constituent amino acids seems to be very similar to that obtained from liver by Bloch *et al.* (1952). No attempt has been made to purify the extract, and it is not possible to say which of the components are essential for the actual building of the peptide and which are concerned with producing the high-energy phosphate bonds necessary to promote the reaction. The fact that HDP, ATP, and DPN cannot be replaced by high concentrations of ATP alone may be due to the very active ATP-ase present in the extracts. Bloch *et al.* (1952) have suggested that in crude extracts

better results may be obtained if the ATP is generated enzymically than if this compound is added to the reaction mixture. Synthesis can occur in the absence of ATP or DPN, but not in the absence of both, and it is possible that DPN may be broken down by the extract to give adenosine phosphates which may be utilized as primers in the HDP fermentation.

Gale & Folkes (1953*b*) have shown that certain antibiotics are potent inhibitors of protein synthesis in staphylococci. None of these antibiotics inhibits the synthesis of glutathione in *Esch. coli*. However, the inhibition studies of Gale were carried out with washed whole-cell suspensions of *Staph. aureus* so that the two systems are not directly comparable. A study of glutathione synthesis in staphylococci might further clarify the problem. The possibility remains that glutathione itself is implicated by transpeptidation reactions in the early stages of protein or peptide formation as suggested by Hanes *et al.* (1950).

SUMMARY

1. Cell-free extracts of *Escherichia coli* synthesize glutathione from glutamic acid, cysteine and glycine in the presence of potassium phosphate, hexose diphosphate, adenosine triphosphate, diphosphopyridine nucleotide and magnesium ions.

2. The results of some kinetic studies on the reaction are given.

3. None of the antibiotics, penicillin, bacitracin, terramycin, aureomycin, chloramphenicol, neomycin, polymyxin, streptomycin, or dihydrostreptomycin, has any effect on the synthesis at concentrations of 100 µg./ml.

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Occurrence of Two Valeric Acids (β -Methylbutyric Acid and α -Methylbutyric Acid) in the Hair Grease of the Dog

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The fatty excretions of the skin of some animals contain not only volatile fatty acids (free and combined) with an even number of carbon atoms, but also volatile fatty acids with an odd number of carbon atoms, in particular valeric acid (Brouwer & Nijkamp, 1952). This acid was found in the 'hair grease' of goats, of sheep, and particularly in that of dogs, where the volatile acid is almost exclusively valeric acid and amounts on an average to 5 % of the

lipid fraction. The question as to which of the isomeric valeric acids occurred was left unanswered, although it was pointed out that isovaleric acid (β -methylbutyric acid) was not unknown in the animal kingdom. Substantial quantities of it occur in the body fat of dolphins and porpoises (Lovern, 1934). A closer investigation has therefore been made into the nature of the valeric acid in the hair grease of the dog.