Levels of glutathione in *Escherichia coli*[1]

PETER C. LOEWEN
Department of Microbiology, University of Manitoba, Winnipeg, Man. Canada R3T 2N2

Received August 16, 1978
Revised September 25, 1978

Loewen, P. C. (1979) Levels of glutathione in *Escherichia coli*. *Can. J. Biochem. 57*, 107–111

Log phase cells of *Escherichia coli* growing in minimal medium contain a basal level of glutathione (5 pmol/mL per Klett unit) which can increase more than sixfold when the cells reach stationary phase. Since the addition of cysteine alone to log phase cells illicits the same response, the increase in the intracellular pool of glutathione appears to be influenced by the amount of cysteine available for glutathione synthesis. Glucose depletion at low cell densities resulted in a decrease in the glutathione pool while the addition of amino acids other than cysteine did not affect the glutathione pool. Depletion of ammonia or proline as the nitrogen source also resulted in a decrease in the glutathione pool to one-third of the original basal level as did a shift to anaerobic growth. The large glutathione pool in stationary phase cells dropped from 31.5 to 4.5 pmol/mL per Klett unit within 30 min of transfer to fresh medium. There was no apparent correlation between changes in the glutathione and coenzyme A – glutathione disulfide (CoASSG) pools after a variety of metabolic disruptions.

Loewen, P. C. (1979) Levels of glutathione in *Escherichia coli*. *Can. J. Biochem. 57*, 107–111

Croissant dans un milieu minimum, les cellules en phase log d'*Escherichia coli* contiennent un niveau basal de glutathion (5 pmol/mL par unité Klett) qui peut augmenter plus de six fois quand les cellules atteignent la phase stationnaire. Comme l'addition de seulement la cystéine aux cellules en phase log produit la même réponse, il semble que l'augmentation du pool intracellulaire du glutathion soit influencée par la quantité de cystéine disponible pour la synthèse du glutathion. La déplétion du glucose à de faibles densités cellulaires entraîne une diminution dans le pool du glutathion, alors que l'addition d'acides aminés autres que la cystéine n'affecte pas le pool du glutathion. La déplétion de l'ammoniaque ou de la proline comme source azotée produit aussi une baisse dans le pool du glutathion qui atteint le tiers du taux basal original, tel qu'il arrive lors du passage à une croissance anaérobie. Le pool important du glutathion, dans les cellules en phase stationnaire, tombe de 31.5 à 4.5 pmol/mL par unité Klett dans les 30 min qui suivent le transfert dans un milieu frais. Aucune corrélation apparente n'est visible entre les changements dans les pools du glutathion et de la coenzyme A – disulfure de glutathione (CoASSG) suite à diverses dégradations métaboliques.

[Traduit par le journal]

Introduction

While glutathione was believed to be present in nearly all types of cells (1), the role of the tripeptide in cellular metabolism was not clearly defined. More recently the importance of glutathione in the cell has been questioned by the finding that many gram-positive bacteria (2) and several mutants of *Escherichia coli* (3, 4, 5) contain no detectable glufathione. Furthermore, the level of glutathione in normal *E. coli* has been shown to vary during growth from a low level in early log phase

ABBREVIATIONS: CoASSG, coenzyme A – glutathione disulfide; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione.

[1]This work has been supported by grant A9600 from the National Research Council of Canada.

0008-4018/79/020107-05$01.00/0

to a high level in stationary phase (2, 3) in spite of the constant specific activity of the glutathione biosynthetic enzymes.

Recently glutathione has been linked to coenzyme A metabolism in *E. coli* by the isolation of CoASSG which forms a major part of the CoA pool in the cell and which was shown to respond to metabolic disruption (6, 7, 8). The present work was undertaken to study the influence of various metabolic changes on glutathione levels in relation to changes previously observed in the CoASSG pool (8) and to test the tentative conclusion (3) that amino acid availability determines the increase in glutathione in stationary phase cells.

Materials and Methods

Materials

Nicotinamide adenine dinucleotide phosphate (reduced form), glutathione, and glutathione reductase (NAD(P)-H: oxidized-glutathione oxidoreductase; EC 1.6.4.2) were obtained from Sigma.

Formic Acid Extracts

Escherichia coli B was grown at 37°C in 50-mL batches of minimal medium in a 250-mL Erlenmeyer flask with an attached sidearm tube for turbidimetric measurement of cell growth using a Klett–Summerson colorimeter. The minimal medium contained 0.1 *M* Tris–HCl pH 8.0, 0.16 m*M* sodium sulphate, 1 m*M* magnesium sulphate, 10 m*M* sodium chloride, 0.2 m*M* calcium chloride, 0.001 m*M* ferric chloride, and 0.5 m*M* potassium phosphate and was supplemented with varying concentrations of carbon source and nitrogen source as noted for each experiment. Aliquots of 5 mL were removed at various times, chilled in a centrifuge tube on ice, and immediately centrifuged. The pellet was frozen at −76°C until all samples from one experiment had been collected. Then the pellets were thawed and resuspended in 0.25 mL of 1 *M* formic acid on ice. After 30 min, the cell debris was removed by centrifugation and the supernatant was neutralized with ammonium hydroxide. One Klett unit is equivalent to 3.6×10^6 cells.

Glutathione Assay

Aliquots of 5–20 μL of the formic acid extracts were assayed for glutathione using the cycling assay of Tietz (9) as modified by Fahey *et al.* (10). All determinations were performed in duplicate at two different extract concentrations. If there was a variation greater than ± 10% among the determinations they were repeated. This procedure measures glutathione both in its reduced and oxidized form although as noted in Ref. 2, very little GSSG was found in *E. coli*. The glutathione and γ-glutamylcysteine dipeptide contents of ^{35}S-labelled cells were compared in one experiment following the labelling and chromatography procedures previously described (4).

Amino Acid Analysis

Aliquots of 50–150 μL of the formic acid extracts were evaporated to dryness and the amino acid content was determined using a Technicon model NC2P amino acid analyzer operated by the Department of Chemistry, University of Manitoba. The determinations were carried out in duplicate with the experimental error ranging from ± 10% for the lower concentrations to ± 2% for the higher concentrations. Since no cysteine or cystine was found in any of the cellular extracts the following control experiment was carried out in duplicate to show that cysteine was not lost during the extraction or analysis procedures. Cysteine (100 nmol) was added to a suspension of cells (from 5 mL of growth medium) in 0.25 mL of 1 *M* formic acid. After 30 min on ice, the cell debris was removed and the formic acid solution was dried by evaporation. The residue was subjected to amino acid analysis which showed 91 and 86 nmol of cysteine to be present in the form of cystine. The acid extraction and evaporation steps result in the oxidation of cysteine to cystine but there is very little loss of the amino acid.

Results

During growth of *E. coli*, the glutathione pool has been reported to increase throughout log phase into stationary phase (3) and to simply increase after stationary phase was reached (2). When the same experiment was repeated in the current work, there was no change in the basal level of glutathione during log phase growth, but upon reaching stationary phase there was a fivefold increase in the glutathione pool, within 1 h (Fig. 1*a*). Quantitation of the amino acid pools showed that glutamic acid increased from 36.7 to 82.3 pmol/mL per Klett unit in the same time period. The glycine pool (2.4 pmol/mL per Klett unit) did not change significantly while the pool of cysteine or cystine could not be quantitated and must have been less than 1.2 pmol/mL per Klett unit. The only other amino acids which were present in appreciable amounts were alanine (15.4 pmol/mL per Klett unit), aspartic acid (3.0 pmol/mL per Klett unit), and serine (2.6 pmol/mL per Klett unit). Since no γ-glutamylcysteine dipeptide was evident in ^{35}S-labelled cells (see also Ref. 3), the rapid synthesis of glutathione involves both γ-glutamylcysteine synthase and glutathione synthase which convert the three amino acids into glutathione as soon as they become available from the shutdown of other metabolic processes.

The available pool of metabolites in stationary phase cells had a significant effect on the synthesis of glutathione. Reducing the concentration of glucose such that cell growth stopped at succeedingly lower cell densities resulted in smaller increases in glutathione. At 0.1% glucose (Fig. 1*b*), 0.067% glucose (not shown), and 0.05% glucose (Fig. 1*c*), there was respectively a 2.5-fold increase, no increase, and a decrease in the glutathione pool. The depletion of both casamino acids and proline as carbon sources resulted in increases in glutathione of 2 and 1.5 times respectively (Fig. 1*d* and 1*e*). These increases occurred at cell densities where glucose depletion had resulted in a reduction in the glutathione pool.

The depletion of both ammonia (Fig. 1*f*) and proline (not shown) as the nitrogen source resulted in a drop in the glutathione pool. Under limiting nitrogen conditions, amino acid biosynthesis would be limited and excess amino acids would not accumulate. Depletion of casamino acids as the nitrogen source, however, resulted in an increase in the glutathione pool (Fig. 1*g*) suggesting that, while some amino acids were limiting growth, glycine, glutamic acid, and cysteine were still present at

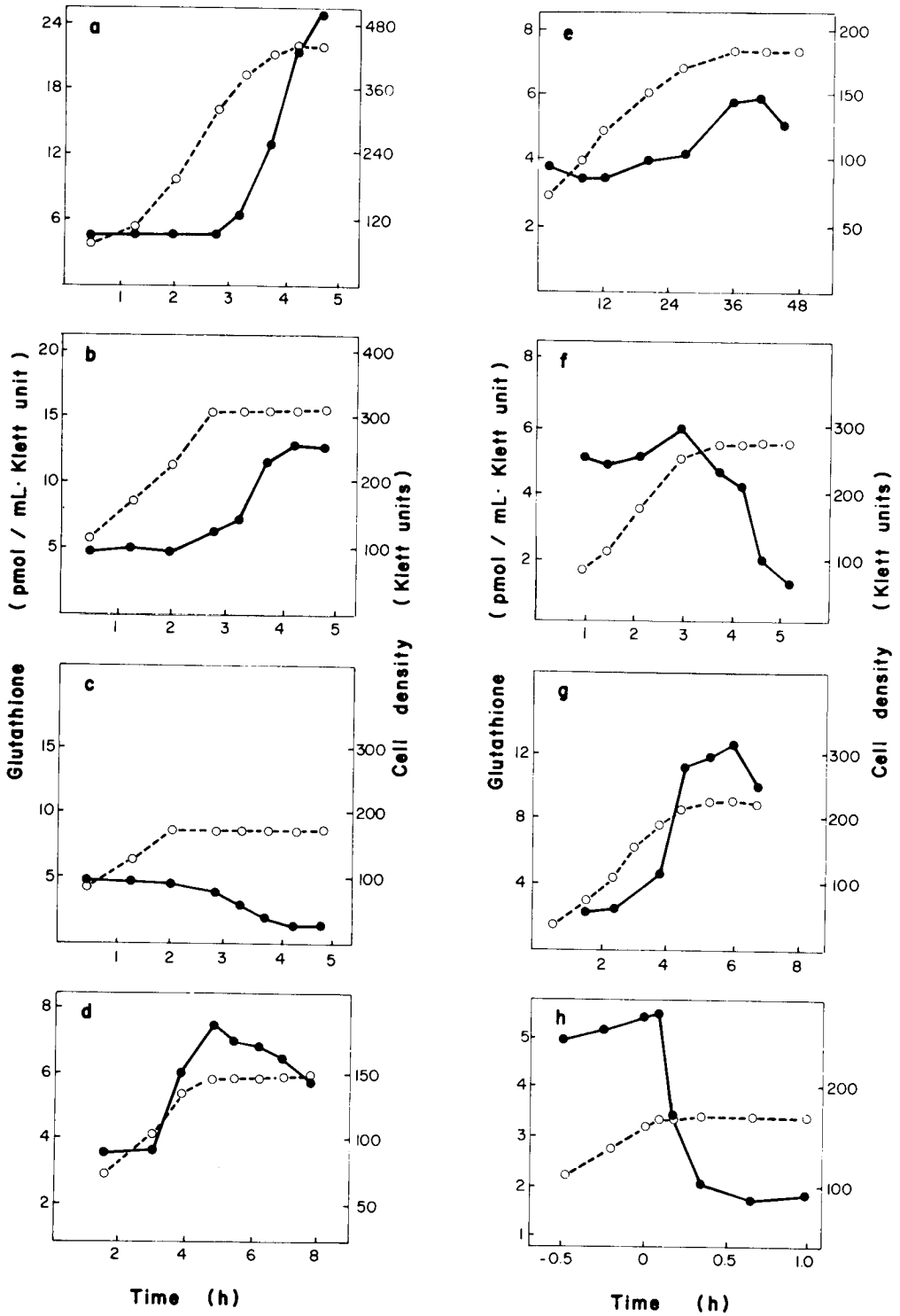

FIG. 1. Glutathione levels in *E. coli* B following various types of starvation. Growth was in minimal medium with the carbon and nitrogen supplements indicated for each experiment: (*a*) 0.2% glucose and 7.5 m*M* ammonium sulphate, (*b*) 0.1% glucose and 7.5 m*M* ammonium sulphate, (*c*) 0.05% glucose and 7.5 m*M* ammonium sulphate, (*d*) 0.2% casamino acids and 7.5 m*M* ammonium sulphate, (*e*) 0.2% proline and 7.5 m*M* ammonium sulphate, (*f*) 0.2% glucose and 1.5 m*M* ammonium sulphate, (*g*) 0.2% glucose and 0.04% casamino acids, and (*h*) 0.2% glucose and 7.5 m*M* ammonium sulphate where aeration was stopped at time 0. Symbols: ●, glutathione; ○, cell density.

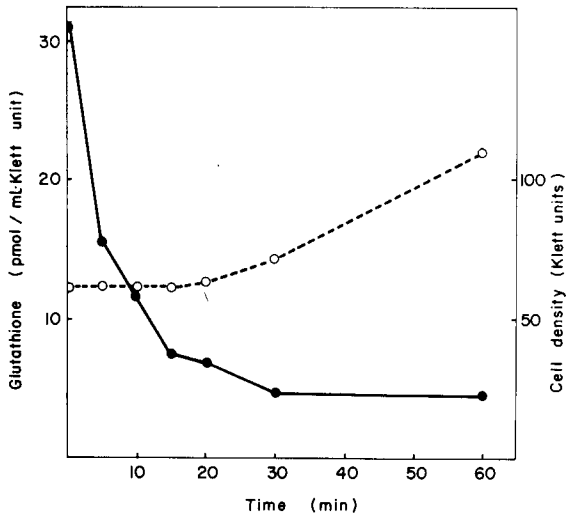

FIG. 2. Glutathione levels in *E. coli* B following dilution of stationary phase cells in fresh minimal medium supplemented with 0.2% glucose and 7.5 m*M* ammonium sulphate. Cells grown 16 h into stationary phase at 37°C in minimal medium were collected by centrifugation and resuspended in fresh medium prewarmed to 37°C. Symbols: ●, glutathione; ○, cell density.

TABLE 1. Glutathione levels 40 min after the addition of various supplements to middle log phase *E. coli* growing in minimal medium containing 0.2% glucose and 7.5 m*M* ammonium sulphate

Supplement	Glutathione, pmol/mL per Klett unit
None	4.9
Cysteine (0.95 μM)	6.7
Cysteine (47.5 μM)	8.7
Cysteine (190 μM)	9.8
Cysteine (1.5 m*M*)	22.9
Casamino acids (0.2% w/v)	15.1
Glutamic acid (0.02% w/v) + glycine (0.02% w/v) + cysteine (0.01% w/v)	11.2
Glutamic acid + glycine (both 0.02% w/v)	4.4
Threonine + proline + histidine + arginine + leucine (all 0.02% w/v)	6.6
Proline (0.1% w/v)	3.3
Cystine (0.01% w/v)	15.4
Mercaptoethanol (10 m*M*)	5.0
Dithiothreitol (1 m*M*)	4.7
Rifampicin (12.5 μg/mL)	2.8
Chloramphenicol (125 μg/mL)	4.8

high enough concentrations to allow glutathione synthesis once that rapid growth had stopped.

The high level of glutathione formed at the onset of stationary phase (Fig. 1*a*) was maintained for at least 8 h. Since early log phase cells contained low levels of glutathione, the glutathione pool must decrease upon transfer of stationary phase cells to fresh medium. As shown in Fig. 2, the expected drop to the basal level

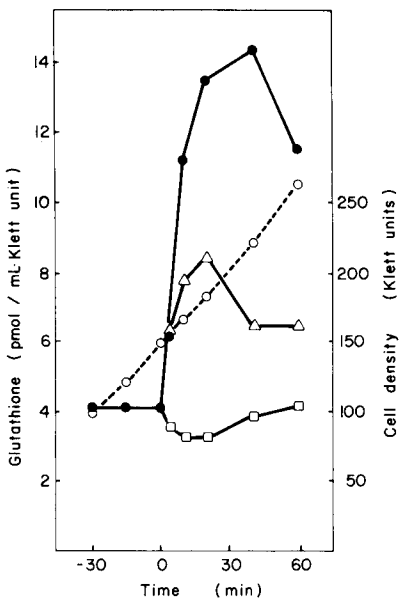

FIG. 3 Glutathione levels in *E. coli* B growing in minimal medium supplemented with 0.2% glucose and 7.5 m*M* ammonium sulphate to which, at time 0, was added: (*a*) ●, 0.01% cysteine; (*b*) △, a mixture of 0.02% glutamic acid, 0.02% glycine, and 0.01% cysteine; or (*c*) □, a mixture of 0.02% glutamic acid and 0.02% glycine. ○, Cell density.

occurred within 30 min and almost 80% of this drop had occurred in the first 15 min after the transfer.

The suggested correlation between the increased availability of amino acids in stationary phase cells and the increase in the glutathione pool was supported by a study of glutathione levels in cells grown in minimal medium supplemented with different combinations of amino acids (Table 1). Middle log phase cells grown in minimal medium supplemented with casamino acids or any amino acid mixture with cysteine as a component contained a larger glutathione pool. Cysteine alone as the supplement resulted in the largest glutathione pool and concentrations of cysteine as low as 0.95 μM allowed an increase in glutathione of 36% over the basal level. Cystine caused a similar increase in the glutathione pool while mercaptoethanol and dithiothreitol did not affect the glutathione pool.

The speed of the response of the glutathione pool to the addition of certain amino acids is shown in Fig. 3. The addition of a glutamic acid – glycine mixture or of each amino acid separately (not shown) resulted in very little change in the glutathione pool. The addition of cysteine resulted in an increase to 3.5 times the basal level within 20 min and some increase was evident within 5 min. Both a glutamic acid – glycine – cysteine mixture and the casamino acid mixture (not shown) promoted smaller increases to two times the basal level with the same rate of response as observed for the addition of cysteine.

The depletion of glucose at low cell densities was

shown to cause a drop in the glutathione pool to 1.5 pmol/mL per Klett unit (Fig. 1*c*). Under the same conditions the basal CoASSG pool has been shown to increase 2.5 times to 0.75 pmol/mL per Klett unit (8). In considering other metabolic disruptions which had caused increases in CoASSG, the glutathione pool was found to decrease or not to change. A shift to anaerobic growth caused a decrease in the glutathione pool to 38% of the original basal level (Fig. 1*h*) and even when the glutathione pool was first raised to 10.44 pmol/mL per Klett unit by the addition of cysteine, the anaerobic shift caused a drop in the glutathione pool to 4.23 pmol/mL per Klett unit or 40% of the original level. Rifampicin caused a decrease in the glutathione pool to 55% of the original level (Table 1) but the other metabolic poisons which affected the CoASSG pool either did not affect the glutathione levels (chloramphenicol) or their effect could not be determined because of significant inhibition of the glutathione assay (KF and NaCN).

Discussion

The observation that the glutathione pool increased only after stationary phase was reached is consistent with one report (2) but not with another report in which the glutathione pool was shown to increase throughout log phase growth (3). There are at least two possible explanations for this discrepancy. Apontoweil and Berends (3) used acid-soluble ^{35}S-labelled radioactivity as their measure of glutathione rather than the direct enzymatic assay used in this work and in Ref. 2. In addition they used a strain of *E. coli* which required that the medium be supplemented with five amino acids and thiamine which may indirectly affect the response of glutathione. In fact Table 1 shows that the five amino acids do elevate the basal level of glutathione.

Since the glutathione biosynthetic enzymes of *E. coli* were shown to be present throughout cell growth at nearly constant specific activity, Apontoweil and Berends (3) proposed that the available amino acid pool determined the size of the glutathione pool. The present work corroborates this hypothesis and shows that it is specifically a low level of cysteine which limits glutathione synthesis in middle log phase cells growing in minimal medium. A basal level of glutathione between 4 and 6 pmol/mL per Klett unit was found in the middle log phase cells but this could vary as low as 1.8 pmol/mL per Klett unit in anaerobic cells or as high as 22.9 pmol/mL per Klett unit when cysteine was present. Both higher and lower limits were observed depending on whether stationary phase was reached at a high or low cell density.

Using the approximate cellular dimensions of $1 \times 1 \times 2.5$ μm for a middle log phase cell, the approximate intracellular concentrations of glutamic acid, glycine, and cysteine are respectively 4.0, 0.3 and less than 0.1 mM. These values can be compared with the K_m values of γ-glutamylcysteine synthase for glutamic acid (1.1 mM) and cysteine(0.8 mM) and of glutathione synthase for glycine (0.4 mM)(3). The pools of glutamic acid and glycine are therefore large enough to satisfy the substrate requirements of the glutathione biosynthetic enzymes leaving cysteine as the limiting precursor. Indeed, concentrations of cysteine in the growth medium as low as 1 μM were sufficient to stimulate glutathione synthesis.

Following the metabolic downshift after growth into stationary phase, there is no increase in any amino acid pool except glutamic acid. Neither glycine nor cysteine pools show any significant change, but in order that the glutathione pool can increase fivefold, both of these amino acids must have become available in amounts much larger than their normal pool size. The regulation of amino acid pool sizes in the cell, however, is such that there is no observable change in the pools in spite of shifts in pool utilization. When the stationary phase cells are diluted in fresh medium, the large pool of glutathione is rapidly depleted prior to the restart of growth possibly as an amino acid source.

There was no apparent correlation between the changes in the CoASSG pool (8) and the changes in the glutathione pool resulting from various metabolic disruptions. Similar conclusions could not be drawn regarding GSSG since its concentration was not determined, but the amount of the disulphide should be much less than the amount of sulphydryl form (2). The CoASSG pool began to increase within 1 or 2 min after an anaerobic shift or the addition of rifampicin, chloramphenicol, KF, and NaCN. The glutathione pool responded more slowly requiring 5 min for any evident change and the only changes observed were decreases after the anaerobic shift and rifampicin addition. The larger size of the glutathione pool makes the independent fluctuation of the two related pools possible. The minimum size observed for the glutathione pool was 1.5 pmol/mL per Klett unit (following glucose starvation) which is two times larger than the maximum CoASSG pool of 0.75 pmol/mL per Klett unit (also following glucose starvation). During normal log phase growth, there is more than a 16-fold difference between the glutathione (4.9 pmol/mL per Klett unit) and CoASSG (0.3 pmol/mL per Klett unit) pools.

1. Kosower, E. M. & Kosower, N. S. (1969) *Nature (London) 224*, 117–120
2. Fahey, R. C., Brown, W. C., Adams, W. B. & Worsham, M.B. (1978) *J. Bacteriol. 133*, 1126–1129
3. Apontoweil, P. & Berends, W. (1975) *Biochim. Biophys. Acta 399*, 1–9
4. Apontoweil, P. & Berends, W. (1975) *Biochim. Biophys. Acta 399*, 10–22
5. Fuchs, J. A. & Warner, H. R. (1975) *J. Bacteriol. 124*, 140–148
6. Loewen, P. C. (1977) *Can. J. Biochem. 55*, 1019–1027
7. Loewen, P. C. (1976) *Biochem. Biophys. Res. Commun. 70*, 1210–1218
8. Loewen, P. C. (1978) *Can. J. Biochem. 56*, 753–759
9. Tietz, F. (1969) *Anal. Biochem. 27*, 502–522
10. Fahey, R. C., Brody, S. & Mikolajczyk, S. D. (1975) *J. Bacteriol. 121*, 144–151