Biochemical Pharmacology, Vol. 36, No. 2, pp. 219–223, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

GLUTATHIONE LEVELS IN CULTURED HEART CELLS

INFLUENCE OF BUTHIONINE SULFOXIMINE, AN INHIBITOR OF GLUTATHIONE SYNTHESIS

WALTER M. LEWKO

Department of Biochemistry and Division of Medical Oncology, James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40292, U.S.A.

(*Received* 4 *February* 1986; *accepted* 28 *May* 1986)

Abstract—In primary cultures of heart cells in mid-growth phase, levels of acid-soluble glutathione were 99 nmoles/mg protein and increased to 178 nmoles/mg protein at confluent growth. Glutathione disulfide accounted for less than 9% of the total. Levels of protein-bound mixed disulfide in mid-growth phase cells were 58 nmoles/mg protein and decreased to 36 nmoles/mg protein at confluent growth. Buthionine sulfoximine (BSO, 10^{-4} M) depleted the levels of both glutathione and glutathione disulfide with no influence on the levels of protein-bound mixed disulfide. BSO had no influence on the multiplication rate of heart cells in primary culture. In secondary passage cultures, the levels of glutathione were less than half those of the primary cultures. BSO depressed cell growth and soluble glutathione, whereas the levels of protein-bound mixed disulfide were increased. These results showed that BSO depletes heart cells of soluble glutathione, whereas protein-bound thiol remains unchanged or increases.

Glutathione (GSH) is a sulfhydryl containing tripeptide and the major non-protein thiol in living systems [1, 2]. Glutathione functions in several cellular processes [1, 2], notably cell division [3, 4], the regulation of free radicals [5] and the metabolism of xenobiotics [6] including certain anticancer drugs [7]. Compounds that alter cellular glutathione levels may be useful in the treatment of cancer [8]. L-Buthionine-*S*,*R*-sulfoximine (BSO) is a compound which depletes cellular glutathione by interfering with the activity of the first of the two enzymes responsible for the synthesis of glutathione [9, 10]. BSO sensitizes cells to the damaging effects of radiation [11] and oxidative cytolysis [12]. BSO is capable of reversing certain forms of drug resistance in human ovarian cancer [13]. In our laboratory, it was observed that BSO depresses the growth rate of several lines of human breast cancer cells [14, *]. In related studies using cultures of human breast cancer cells, BSO appeared to stimulate the activities of the anticancer drugs *cis*-platinum and doxorubicin (unpublished results).

It was, therefore, of interest to determine the effects of BSO on growth and the levels of glutathione in cultures of normal cells for comparison with cancer cells. Heart cells were chosen for this study. Several good techniques have been developed for the dispersion of heart cells for growth in culture. Moreover, heart tissue is particularly sensitive to reactive oxygen metabolites [15]. Since there is evidence that glutathione may be involved in protecting cardiac tissues against reactive oxygen [15, 16], it was of interest to determine whether heart cells were particularly sensitive to the potential toxic effects of glutathione depletion by BSO. In addition to measuring the effects of BSO on cell growth and the levels of acid soluble glutathione, protein-bound mixed disulfide (PSSR) was measured. It has been suggested that glutathione in the form of protein-bound mixed disulfide may represent a slowly available GSH pool [1].

* W. M. Lewko, *13th Int. Congr. Biochem.*, Abstract, p. 51 (1985).

MATERIALS AND METHODS

Primary cultures of rat heart cells were prepared by a modification of the method published by Harary and Farley [17]. Sprague–Dawley rats (2- to 4-days-old) were killed by cervical dislocation. Hearts were excised and rinsed with sterile phosphate-buffered saline. Ventricular tissue (0.7 g) was coarsely minced with scissors and suspended in 15 ml of dispersion solution which was composed of calcium- and magnesium-free Earle's balanced saline with 0.125% Trypsin (T 8253, Sigma Chemical Co., St. Louis, MO). The heart tissue was finely minced by passing it, three times, through a Tekmar Tissumincer which was operating at low speed (control setting 30). The heart tissue was digested with slow stirring for 1 hr at 37° in a CO_2 incubator. Then 0.1 mg of DNase (104, 159, Boehringer Mannheim Biochemicals, Indianapolis, IN) was added, and the dispersed cells were passed through a 125 micron mesh nylon filter (Martin Supply Co., Baltimore, MD). The cells in the filtrate were suspended in culture medium consisting of Eagle's Minimum Essential Medium (MEM) (410-1100, Gibco, Grand Island, NY), 5% fetal calf serum (JR Scientific, Woodland, CA), and gentamycin (40 μg/ml, Sigma Chemical Co.) and plated on tissue culture dishes. The cultures were maintained at 37° in an incubator with an atmosphere of 95% air and 5% CO_2. After 18–24 hr, the unat-

tached cells (predominantly erythrocytes) were rinsed away as the plating medium was replaced with fresh culture medium. A small number (less than 1%) of the cells in primary culture were beating. The heart cell cultures were passed by standard trypsinization procedures.

Glutathione was measured in acid extracts of cultured cells. Cells were quickly rinsed with cold phosphate-buffered saline, lysed in 5% (w/v) metaphosphoric acid (Aldrich Chemical Co., Milwaukee, WI), scraped from the dish, and sonicated. The samples were then centrifuged for 10 min at 13,000 *g* in a Fisher 235 Microfuge. Total acid-soluble glutathione was measured in supernatant fractions by the enzymatic technique of Tietze [18]. Glutathione disulfide (GSSG) was measured similarly after blocking reduced glutathione with 2-vinylpyridine as described by Griffith [19]. Protein-bound mixed disulfide was estimated in the 5% metaphosphoric acid cell protein precipitates by solubilizing and reducing each precipitate in a solution containing 8 M guanidine and 2 mg/ml sodium borohydride as described by Akerboom and Seis [20] and Modig [21]. Released sulfhydryl was measured in supernatant fractions of reacidified samples using the fluorometric *o*-phthaldialdehyde method of Cohn and Lyle [22]. All values are reported in terms of nanomole equivalents of reduced glutathione.

Cellular protein was measured using the colorimetric method of Bradford [23]. Bovine serum albumin was used as a standard. DNA was determined by the fluorometric bisbenzimide (Boehringer Mannheim Biochemicals) method as described by Downs and Wilfinger [24] using calf thymus DNA as a standard.

L-Buthionine-*S*,*R*-sulfoximine (BSO) was purchased from the Chemical Dynamics Corp., South Plainfield, NJ. Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)], *o*-phthaldialdehyde, NADPH (Type III), reduced glutathione and glutathione reductase (Type III) were purchased from the Sigma Chemical Co.

Statistical significance was assessed by analysis of variance. Data were fit to linear models, $Y_1 = b_0 + (b_1 + b_2)\ T_1$ and $Y_2 = b_0 + b_2 T_2$, where Y_1 and Y_2 refer to the response of two sets of cultures with time T, each treated with different concentrations of BSO; b_0 is a constant (Y-intercept); b_1 is the slope for the data representing the higher drug concentration and b_2 is the difference between the two compared slopes. Statistical significance for a slope, positive or negative, indicated significant change in a measured parameter of the culture with time. Statistical significance for the difference between slopes, b_2, indicated altered response between experimental cultures.

RESULTS AND DISCUSSION

Heart cells plated in growth medium attached within 18 hr and then proceeded to multiply and grow out over the culture surface. Initially, the heart cells exhibited a doubling time of approximately 24 hr. When viewed on day 3 by phase contrast microscopy, there appeared to be two basic types of cells. The first consisted of flattened cells with multiple processes. These cells appeared to be firmly attached and spreading. The second consisted of cells which were elongate, with one or two processes, frequently spindle-like in shape. (Beating heart cells in these primary cultures were of the elongated cell type.) At a concentration of 10^{-4} M, BSO did not have any influence on the morphology of cells in primary cultures (data not shown).

Glutathione levels measured in primary cultures, were expressed per culture and in terms of cellular protein and DNA. As the cells grew, the levels of reduced glutathione (Fig. 1) and glutathione disulfide (Fig. 2) increased with time. Glutathione disulfide accounted for less than 9% of the total soluble glutathione. When buthionine sulfoximine was added to the culture medium, the levels of reduced glutathione decreased to less than 5% within 24 hr (Fig. 1). The levels of glutathione disulfide decreased more gradually with time (Fig. 2). The average percentage of glutathione disulfide tended to be higher in the BSO-treated versus untreated cultures.

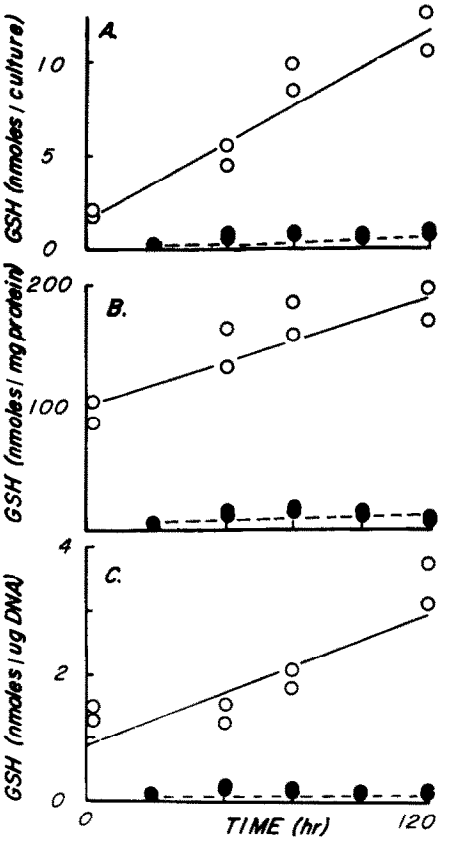

Fig. 1. Glutathione (GSH) levels in primary cultures of rat heart cells. Cells were cultured in growth medium (solid line, ○, control) or in medium containing BSO (broken line, ●, 10^{-4} M). At the indicated time points, cellular extracts for GSH analysis were prepared from duplicate control and BSO-treated cultures. Results were expressed per culture (A), per mg protein (B) and per μg DNA (C). Lines were drawn from the linear regression of the indicated points. In each case, the slopes of lines representing controls were significantly positive and statistically different from the slopes derived from cultures treated with BSO ($P < 0.005$).

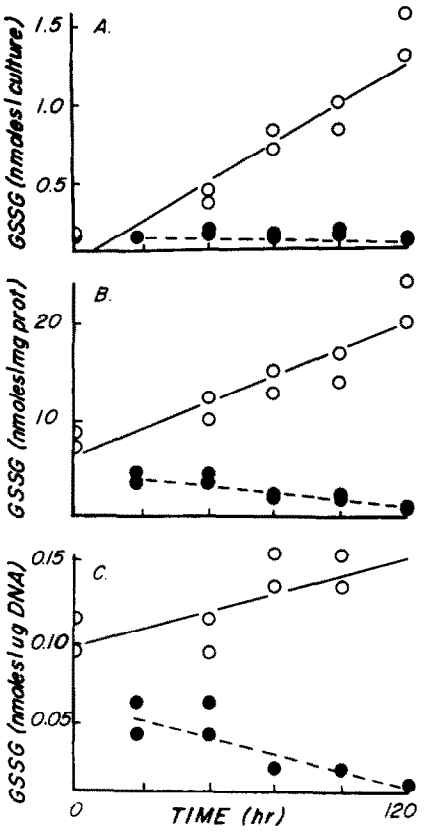

Fig. 2. Glutathione disulfide (GSSG) levels in primary cultures of rat heart cells. Cells were cultured in growth medium (solid line, ○, control) or in medium containing BSO (broken line, ●, 10^{-4} M). At the indicated times, cellular extracts from GSSG analysis were prepared from duplicate control and BSO-treated cultures. Results were expressed per culture (A), per mg protein (B) and per μg DNA (C). The slopes were significantly positive in the case of the untreated cultures and negative in the case of the BSO-treated, with one exception, the BSO-treated, panel A. In each case, the slopes of lines for the untreated were significantly different from the slopes for cultures treated with BSO ($P < 0.005$).

Protein-bound mixed disulfide (PSSR) was estimated by measuring the acid-soluble thiols released as proteins were reduced with sodium borohydride. When expressed per culture, the content of protein-bound mixed disulfide (Fig. 3) increased over the first 72 hr of the experiment. When expressed in terms of protein and DNA, the relative amounts of PSSR decreased with time as the cultures approached confluence. The addition of 10^{-4} M BSO to the culture medium did not have any significant influence on protein-bound thiol (Fig. 3). The percent of total cellular thiol in the form of protein-bound mixed disulfide increased in the BSO-treated cultures relative to soluble glutathione.

Depletion of soluble glutathione and glutathione disulfide did not appear to be toxic to the primary cultures over the 5-day period of this study. There were no obvious differences in morphology comparing the control and treated cultures. BSO had no influence on cell multiplication as indicated by the levels of culture DNA (Fig. 4B). Interestingly, cell protein tended to be higher in the BSO-treated cultures (Fig. 4A).

The morphology and growth rates of second passage heart cell cultures differed considerably from those of primary cultures. The secondary cultures consisted of very flattened, well-spread cells with relatively small processes. The spindle-shaped and beating heart cells characteristic of the primary cultures were not observed. The growth rate of these cells was remarkably slow with an estimated doubling time of only 5–6 days. Further, the levels of soluble glutathione (oxidized and reduced) were relatively low in second passage cultures (Fig. 5). In response to the addition of BSO to the culture medium, levels of soluble glutathione decreased with time (Fig. 5). The levels of protein-bound mixed disulfide increased in a time- and dose-related manner (Fig. 6).

The influence of BSO on cell growth is shown in Fig. 7. In contrast to the lack of a growth effect in primary cultures, BSO appeared to decrease the

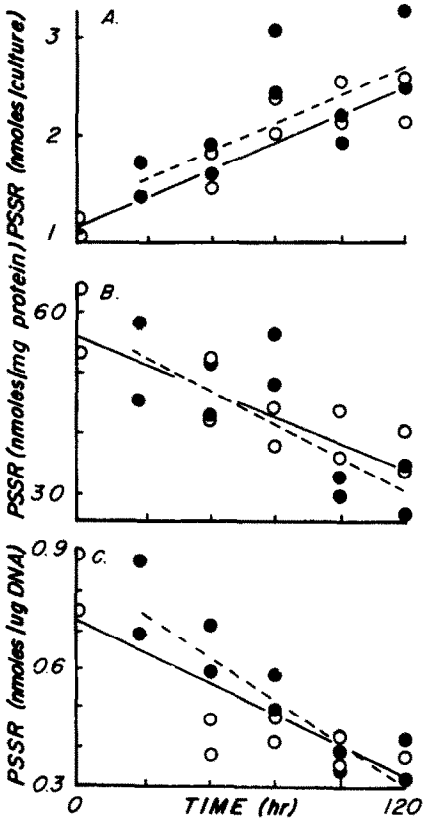

Fig. 3. Protein-bound mixed disulfide (PSSR) in primary cultures of rat heart cells. Cells were cultured in growth medium (solid line, ○, control) or in medium containing BSO (broken line, ●, 10^{-4} M). At the indicated times, cellular extracts were prepared for PSSR analysis. Slopes for control and BSO-treated cultures were significantly positive when expressed per culture (A), and significantly negative when data were expressed per mg protein (B) and per μg DNA (C). When the results for control and BSO-treated cultures were compared, differences in the slopes were not statistically significant, indicating that PSSR levels were not different in the BSO-treated heart cells.

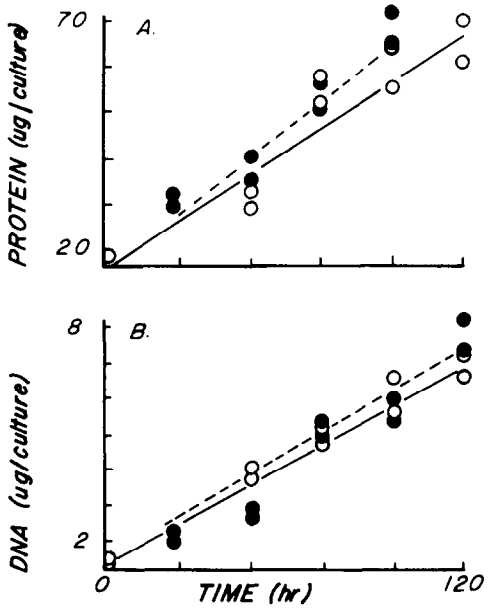

Fig. 4. Cell growth in primary cultures of rat heart cells. Heart cells were plated on 60 mm dishes. Two days later, the plating medium was replaced with fresh growth medium (solid line, ○, control) or medium which contained BSO (broken line, ●, 10^{-4} M). At the indicated times, cultures were taken for the analysis of protein (A) and DNA (B). The slope of each line was significantly positive, indicating that growth occurred in the cultures. When the slopes obtained from the DNA analyses were compared, there were no statistically significant differences between control and BSO-treated cultures, indicating no difference in cell number. When the slopes obtained from protein analyses were compared, the slope of the BSO treated cultures was higher than that of the control ($0.05 < P < 0.01$).

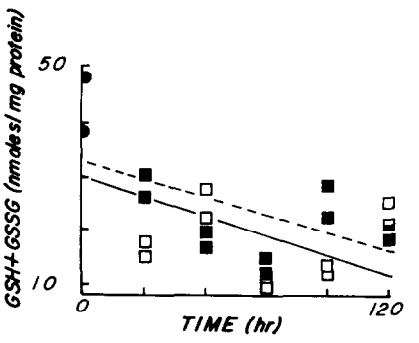

Fig. 5. Glutathione (GSH + GSSG) in second passage cultures of rat heart cells. Heart cells were cultured for a total of 17 days. BSO was added to the culture medium at concentrations of 10^{-4} M (solid line, □) or 10^{-3} M (broken line, ■) for the number of days indicated. Untreated cultures are represented by closed circles. All cultures were terminated on day 17, and extracts were prepared for the analysis of glutathione. Correlation coefficients for the two lines were 0.55 and 0.64 respectively. The slopes of the lines were significantly negative ($P < 0.005$), indicating a decrease in glutathione with time. When compared, the slopes of the lines representing the two concentrations of BSO were not statistically different.

growth rate of cells in the second passage cultures. After 5 days, the cell protein values of cultures treated with 10^{-4} M and 10^{-3} M BSO were 76 and 45% of control untreated cultures respectively. By phase contrast microscopy, the BSO-treated cultures were different morphologically (less spread) after 4 days in both treatment groups. There was no evidence for BSO-induced cell detachment.

Buthionine sulfoximine is effective experimentally in potentiating the anticancer effects of radiation and certain forms of chemotherapy [11, 13]. BSO depressed the growth of primary cultures of rat mammary tumor cells and several types of cultured human breast cancer cells [14, *]. BSO caused the depletion of glutathione in these cells, an effect which appeared responsible for its growth-inhibiting properties. BSO is an inhibitor of the enzyme γ-glutamylcysteine synthetase, the first of two enzymes directly involved in the synthesis of glutathione [9–11]. Depletion occurs as glutathione is metabolized or transported out of the cell. Any differences in the rates at which glutathione is utilized would influence the rate at which glutathione is depleted in BSO-treated cells. Differential sensitivity of tumor and normal tissues with respect to glutathione depletion and glutathione requirements serves as a basis for the value of BSO in anticancer therapy [8]. Under similar culture conditions, breast cancer cells contained 30–60 nmoles glutathione per mg protein. However, the levels of glutathione appear to be more stable in breast tumor cells since 24–48 hr are required for maximal BSO effect [14, *]. This is somewhat longer than the time required to depress glutathione in the heart cell cultures which responded with near maximum depletion within 22 hr. However, with respect to growth inhibition, heart cells were less sensitive to BSO than tumor cells. In cultures of human MCF-7 and MDA-231 cells, BSO at 10^{-4} M over a 5-day treatment period caused greater than 50% inhibition of growth. Under similar culture conditions, BSO was less effective in depressing the growth of secondary heart cultures and had no inhibitory effect at all on the

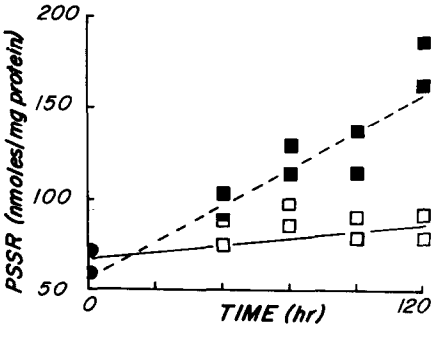

Fig. 6. Protein-bound mixed disulfide (PSSR) in second passage cultures of rat heart cells. Heart cells were cultured and treated with BSO as described in Fig. 5. The slope of each line was significantly positive ($P < 0.005$), indicating increased PSSR with time in cultures treated with BSO. The difference between the slopes was significant ($P < 0.005$), indicating that PSSR accumulated more quickly in cultures treated with the higher dose of BSO.

* W. M. Lewko, *13th Int. Congr. Biochem.*, Abstract, p. 51 (1985).

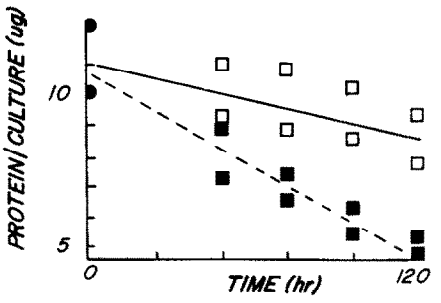

Fig. 7. Influence of BSO treatment on cell growth in second passage cultures of heart cells. Cultures were treated for 0–5 days with growth medium containing BSO at 10^{-4} M (solid line, □) or 10^{-3} M (dashed line, ■) as indicated in Fig. 5. Cellular protein was measured in duplicate cultures. Untreated cultures are represented by closed circles. The slope of each line was significantly negative ($P < 0.005$), indicating decreased cellular protein with time in cultures treated with BSO. The difference between the slopes was significant ($P < 0.005$), indicating that protein levels were lower in cultures treated with the higher dose of BSO.

growth of primary heart cultures. BSO may be of value as an anticancer agent; however, care must be exercised in the development of protocols, particularly those involving drugs, such as doxorubicin, which exhibit glutathione-sensitive cardiotoxic effects [16].

Acknowledgements—I would like to thank Danny E. Winn for his technical assistance and Judith Theriault-Lewko and Dr. Martin D. Williams for their comments during the preparation of this manuscript. I would also like to thank Drs. John Richie, George Hazelton, Betty Jane Mills and Calvin Lang for their advice regarding the glutathione assays. I am particularly grateful to Dr. Richard Greenberg for his recommendations on the statistical interpretation of the data. This work was funded in part by PHS Grant CA 32240 awarded by the National Cancer Institute, DHHS and by a grant from the Graduate School, University of Louisville.

REFERENCES

1. N. S. Kosower and E. M. Kosower, *Int. Rev. Cytol.* **54**, 106 (1978).
2. A. Meister and M. E. Anderson, *A. Rev. Biochem.* **52**, 711 (1983).
3. M. Luthman, S. Eriksson, A. Holmgren and L. Thelander, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2158 (1979).
4. D. Mazia, in *Glutathione* (Eds. S. Colowick, A. Lazarow, E. Racker, D. R. Schwarz, E. Stadtman and H. Welsch), p. 209. Academic Press, New York (1954).
5. G. Cohen and P. Hochstein, *Biochemistry* **2**, 1420 (1963).
6. L. F. Chausseaud, *Adv. Cancer Res.* **29**, 175 (1973).
7. B. A. Arrick and C. F. Nathan, *Cancer Res.* **44**, 4224 (1984).
8. A. Meister and O. W. Griffith, *Cancer Treat. Rep.* **63**, 1115 (1984).
9. O. W. Griffith and A. Meister, *J. biol. Chem.* **254**, 7558 (1979).
10. O. W. Griffith, *J. biol. Chem.* **257**, 13704 (1982).
11. J. K. Dethmers and A. Meister, *Proc. natn. Acad. Sci. U.S.A.* **78**, 7492 (1981).
12. B. A. Arrick, C. F. Nathan, O. W. Griffith and Z. A. Cohn, *J. biol. Chem.* **257**, 1231 (1982).
13. J. A. Green, D. T. Vistica, R. C. Young, T. C. Hamilton, A. M. Rogan and R. F. Ozols, *Cancer Res.* **44**, 5427 (1984).
14. W. M. Lewko, D. E. Winn and K. P. McConnell, *Proc. Am. Ass. Cancer Res.* **26**, 39 (1985).
15. J. H. Doroshow, C. Y. Locker and C. E. Myers, *J. clin. Invest.* **65**, 128 (1981).
16. R. D. Olson, J. S. MacDonald, C. J. van Boxtel, R. C. Boerth, R. D. Harbison, A. D. Slonim, R. W. Freeman and J. A. Oates, *J. Pharmac. exp. Ther.* **215**, 450 (1980).
17. I. Harary and B. Farley, *Expl Cell Res.* **29**, 451 (1963).
18. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
19. O. W. Griffith, *Analyt. Biochem.* **106**, 207 (1980).
20. T. P. M. Akerboom and H. Seis, *Meth. Enzym.* **77**, 373 (1981).
21. H. Modig, *Biochem. Pharmac.* **17**, 177 (1968).
22. V. H. Cohn and J. Lyle, *Analyt. Biochem.* **14**, 434 (1974).
23. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
24. T. R. Downs and W. W. Wilfinger, *Analyt. Biochem.* **131**, 538 (1983).