AN ULTRASENSITIVE ASSAY FOR SOLUBLE SULFHYDRYL AND ITS APPLICATION TO THE STUDY OF GLUTATHIONE LEVELS DURING THE HELA LIFE CYCLE

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Changing glutathione levels have often been implicated in mitotic events since Rapkine discovered the cyclic variation of a trichloroacetic acid (TCA)-soluble sulfhydryl (SH)-containing molecule in synchronously dividing sea urchin embryos (1). However, Waley has pointed out that many studies of glutathione fail to use assays of sufficient specificity, in that small peptides, ascorbate, and cysteine frequently lead to ambiguous measurements (2); indeed, reexamination of the Rapkine cycle, at least for sea urchin, has disclosed that the variation in TCA-soluble SH content is due to a small peptide other than glutathione (3). In order to examine glutathione levels in a mammalian cell during its life cycle, we have developed a simple, specific, and comparatively sensitive procedure (see 4 for review) applicable to samples as small as 0.5 mg wet weight. Briefly, the 100,000 *g* supernate from a cell lysate is reacted with *N*-ethylmaleimide-^{14}C (NEM). The reaction with SH involves addition of a sulfanion to the double bond of the imide in NEM and is specific for the SH grouping at pH 7 over a 15 min interval (5). The glutathione adduct is then isolated from other SH-containing (radioactive) species by acrylamide gel electrophoresis and quantitated by scintillation counting. States and Segal have also used NEM-^{14}C to separate the glutathione adduct from a complex biological system on thin-layer chromatography; however, no attempt at quantification was made (6).

MATERIALS AND METHODS

Glutathione Assay

Reduced glutathione (GSH) standards were freshly prepared in 0.01 M PO_4 buffer (pH 7.4) with 0.1% sodium dodecyl sulfate (SDS) and adjusted so that 0.25 ml contained 2-100 mμmole (10^{-9} M); 0.25-ml aliquots were mixed with 0.05 ml of *N*-ethylmaleimide-^{14}C containing 0.5 μCi (specific activity 10.3 mCi/mmole) and the reaction was allowed to proceed at 21°C for 15 min. Sample volumes of 0.30 ml were placed on 7.5% polyacrylamide gels prepared as previously described (7) and run at 21°C for 3 hr at 12 ma/gel. The gels were mechanically fractionated (8), mixed with Bray's fluid, and counted in a Packard Tri-Carb spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Similar gels were run with the NEM-cysteine reaction product and with NEM alone.

Extraction of Ethanol-Soluble SH Fraction

50 ml of HeLa cells (3 mg/ml) were labeled for 3 hr with cystine-^{3}H or leucine-^{3}H (1 μCi/ml) and then washed twice with Earle's salt solution containing 0.5% Carbowax (Union Carbide Corp., New York), addition of which markedly decreased the fraction of visibly disintegrating cells during the wash procedure. 1 ml of 95% ethanol was

added to the cell pellet, and the insoluble residue was removed by centrifugation. The extract was reduced to about 0.05 ml under a stream of N_2. Following dilution to 0.4 ml with the PO_4-SDS buffer, the extract was reacted with 0.1 ml of 10^{-3} M NEM; 0.3 ml samples were electrophoresed, fractionated, and counted as above. The radioelectrophoretic patterns were compared with those obtained from standard GSH-NEM. In some experiments isotopic NEM-^{14}C was used to label the SH moiety.

Application of Glutathione Assay to Synchronized HeLa Cells

HeLa cells (S_3 strain) were routinely maintained in Eagle's spinner medium plus 7% fetal calf serum as previously described (9). Synchronized populations of mitotic cells were harvested by selective detachment from monolayer cultures grown in low calcium medium (10) and either were used for glutathione analysis immediately or were placed in spinner culture and allowed to proceed into interphase. Progression of the population through the cell cycle was monitored by thymidine-^{14}C incorporation, and cells were processed during G_1 (postmitotic phase) and S as well as during mitosis. Aliquots were rinsed twice in cold Earle's salts containing 0.5% Carbowax. Packed cell volumes were determined in specially designed graduated capillary tubes and converted to cell mass after appropriate correction for trapped extracellular fluid (11). Samples were rinsed from the capillary tubes with either 0.5 ml of 95% ethanol or 0.5 ml of cold hypotonic buffer (0.001 M, PO_4, pH 7.4, 0.001 M, $MgCl_2$). Ethanol extracts were treated as above. Cells swollen in hypotonic buffer were allowed to stand for 5 min at 4°C. They were then disrupted with a Dounce-type homogenizer, and the homogenate was centrifuged at 40,000 rpm in the Spinco 40 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) for 15 min at 4°C. The supernate was made to 0.01 M PO_4 and 0.1% SDS; NEM-^{14}C was added to a final concentration of 2 μCi/ml and the reaction was allowed to proceed as above. The supernate from 0.5 to 2 mg of cells was placed on polyacrylamide gels, electrophoresed, and processed as described. In some experiments the sample was dialyzed before electrophoresis.

RESULTS AND DISCUSSION

Fig. 1 shows that the NEM-glutathione product is detected as a discrete, rapidly migrating peak of radioactivity on polyacrylamide gels. NEM-cysteine runs much more slowly in the buffer system employed, and NEM alone (not plotted) gives a broad smear with no peak at all. The peak

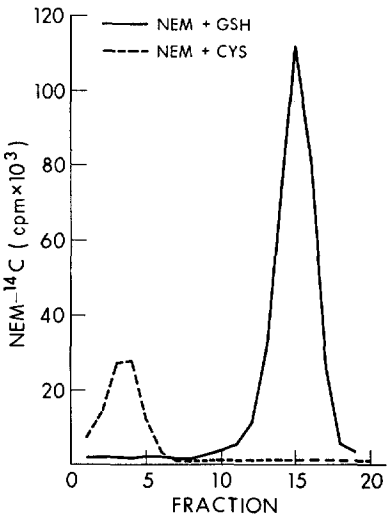

FIGURE 1 Gel electropherogram of NEM-GSH and NEM-cysteine adduct; 35 × 10^{-3} μmoles of GSH or 30 μmoles of cysteine in 0.25 ml of PO_4-SDS buffer was reacted for 15 min at 21°C with slight excess NEM (approximately 200 × 10^{-3} μmoles). A 0.30 ml volume was electrophoresed on polyacrylamide gels as described in the text. NEM alone (50 × 10^{-3} μmoles) was also run but not plotted.

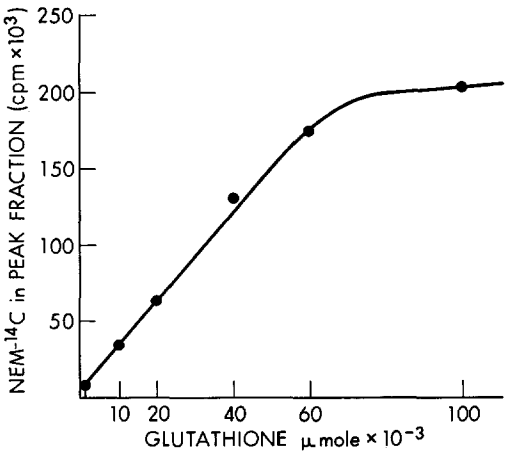

FIGURE 2 Curve showing region of linear relationship between peak height of GSH-NEM adduct and concentration of GSH. Data derived from experiments such as that described in Fig. 1.

height of 1.15 × 10^5 cpm obtained on 35 × 10^{-3} μmoles of glutathione emphasizes the sensitivity of the method. Fig. 2 shows that there is linearity of peak height with glutathione concentration between 2 and 60 × 10^{-3} μmoles. Above 2 × 10^{-4}

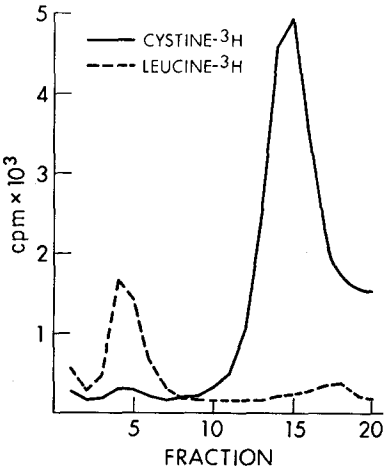

FIGURE 3 Gel electropherogram of ethanol extract from HeLa cells labeled with cystine-^{3}H or leucine-^{3}H as described in the text. Ethanol was evaporated and sample was redissolved in PO_4-SDS buffer, reacted with excess NEM (nonradioactive), and electrophoresed on polyacrylamide gels.

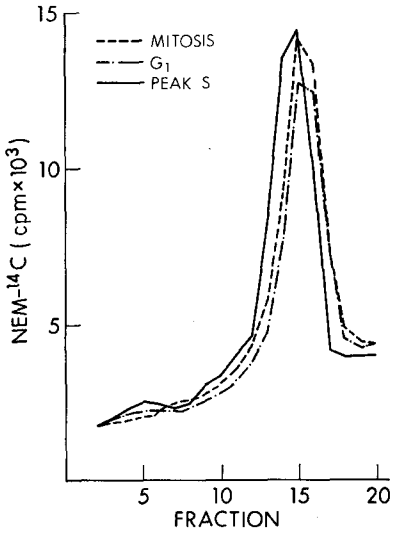

FIGURE 4 Electropherograms of NEM-reacted 100,000 *g* supernatant fraction from synchronized cells. Populations in G_1, S, and mitosis were swollen in hypotonic buffer, and mechanically broken. The 100,000 *g* supernatant fraction was collected and made 0.01 M in PO_4, 0.1% in SDS. Excess NEM-^{14}C was added as above, and the samples were electrophoresed. Although not plotted, several time points were taken in both G_1 and S.

molar concentration (60×10^{-3} μmoles/0.30 ml sample), the counts plateau. This is in reasonable agreement with the calculated input of NEM (50×10^{-3} μmoles/0.30 ml sample).

When HeLa cells are labeled with cystine-^{3}H or leucine-^{3}H and the ethanol extract is then reacted with NEM-^{14}C, the resulting electropherograms appear as in Fig. 3. There is a large peak in the ethanol extract which contains cystine but not leucine, and which runs in exactly the same location as the NEM-GSH adduct. The bound NEM counts are not plotted to avoid confusion but, as expected, they run with the cystine counts. The leucine-deficient, cystine-rich, NEM-reactive character of the more prominent ethanol-soluble peak along with its glutathione equivalent electrophoretic mobility, and the fact that we find that the peak disappears if the sample is dialyzed before electrophoresis strongly indicate that it is glutathione. A similar peak was obtained when samples were prepared from the 100,000 *g* supernate of HeLa cells lysed in hypotonic buffer. Although this latter method gave more background the results were, in general, more quantitative, and it was used routinely in the study of glutathione levels in synchronized cells. It should be mentioned that assay with bromphenol blue established that the NEM-GSH adduct did not run at the gel front but rather considerably behind it, and consequently our results do not represent artifactual stacking of fast running peptides.

Fig. 4 is a composite of three electropherograms made from *equal masses* of HeLa cells at the indicated time points in the life cycle. It is clear that there is very little difference between the GSH-NEM peaks yielded by cells in mitosis and those from cells in G_1 or S. The 2–3000 cpm background level of NEM-^{14}C which smears across the early gel fractions is due to both the labeled NEM that passively enters the gel and to those SH-containing proteins present in the 100,000 *g* fraction. However, no other peak containing NEM activity is quantitatively significant compared to that which we assume to be the NEM-GSH adduct.

The approximately 10% drop in glutathione levels noted between mitosis and early G_1 is thought to be insignificant; while it is fairly reproducible, there is some evidence that it may be a delayed response to the environmental perturbations that the cells necessarily experience at the time of synchronization. In any case, we can state with a fair degree of certainty that, if any variation in glutathione levels occurs during the cell cycle, it

is small. This is in accord with the findings of Sakai and Dan in sea urchin (3).

While the above data establish the relative constancy of glutathione during the HeLa cell cycle, they give no indication of what role (if any) glutathione might play in cell division; the the role of SH in general with respect to cell division remains obscure; however, the present methodology does allow detailed investigation of mammalian SH-containing polypeptides, and it will be of considerable interest to determine whether any of them follow the cyclic variation of the TCA-soluble protein described by Sakai and Dan (3).

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REFERENCES

1. RAPKINE, L. 1931. *Ann. Physiol., Par.* **7**:382.
2. WALEY, S. F. 1966. *Advan. Protein Chem.* **21**:5.
3. SUKAI, H., and K. DAN. 1959. *Exp. Cell Res.* **16**:24.
4. PATTERSON, J. W., and S. LAZAROW. 1954. *In* Glutathione. S. Colowick, A. Lazarow, E. Racker, D. Schwarz, E. Stadtman, and H. Waelsch, editors. Academic Press Inc., New York. 63
5. GREGORY, J. D. 1955. *J. Amer. Chem. Soc.* **77**:3922.
6. STATES, B., and S. SEGAL. 1969. *Anal. Biochem.* **27**:323.
7. MAIZEL, J. V. 1969. *In* Fundamental Techniques in Virology. K. Habel and N. Salzman, editors. Academic Press Inc., New York. 334.
8. MAIZEL, J. V. 1966. *Science (Washington).* **151**:988.
9. EAGLE, H. 1959. *Science (Washington).* **130**:432.
10. ROBBINS, E., and M. D. SCHARFF. 1966. *In* Cell Synchrony. I. Cameron and E. Padilla, editors. Academic Press Inc., New York.
11. MORRILL, G. A., and E. ROBBINS. 1967. *J. Gen. Physiol.* **50**:782.