VARIATIONS IN SULFHYDRYL, DISULFIDE, AND PROTEIN CONTENT DURING SYNCHRONOUS AND ASYNCHRONOUS GROWTH OF HeLa CELLS

F. MAURO, A. GRASSO, *and* L. J. TOLMACH

From the Division of Radiation Biology, Department of Radiology, School of Medicine; the Department of Biology; and the Committee on Molecular Biology, Washington University, St. Louis, Missouri 63110

ABSTRACT The cellular contents of protein-bound and nonprotein sulfhydry (—SH) and disulfide (—SS—) groups were measured in both asynchronous and synchronous HeLa S3 cultures. About 90% of these groups are associated with proteins, the majority in the —SH form. The content of protein-bound groups, and hence the total content of —SH and —SS— groups (28×10^{-15} moles/cell, or 1.1×10^{-6} moles/g protein on average), changes in parallel with the protein content (which varies between 2 and 4×10^{-10} g/cell) as asynchronous populations pass from the lag through the exponential to the stationary phase of growth. The concentration of nonprotein —SH groups, in contrast, increases 10-fold during lag phase and decreases in stationary phase; it follows the protein concentration closely during the exponential phase, at a level of about 2.8×10^{-15} moles/cell. In synchronous cultures the protein content doubles during the cell cycle, possibly in an exponential fashion. The total —SH and —SS— content also doubles, but the rate of increase appears to fluctuate. The concentrations of the protein-bound groups show 2- to 3-fold fluctuations per unit protein: protein-bound —SH groups and mixed —SS— linkages rise to maxima while protein-bound —SS— groups fall to a minimum at the G_1/S transition, and fluctuations in these groups occur again during G_2. In addition, the protein-bound —SH concentration falls continuously during the S phase. The nonprotein —SH concentration undergoes the largest (relative) fluctuations, dropping from 4×10^{-15} moles/cell in early G_1 to about 0.4×10^{-15} moles/cell (of standard protein content) at the end of G_1, and then rising to 30 times this value by the end of S.

INTRODUCTION

After the initial observation by Howard and Pelc (1) that nuclear DNA synthesis in the cells of *Vicia faba* occurs only during a discrete part of interphase (S phase), and subsequent demonstrations that a similar pattern of DNA synthesis is general in plant and animal cells, the syntheses of other cellular constituents have

been shown to fluctuate in a regular fashion during the cell cycle. Cultured animal cells have served as experimental material in many of these studies, partly because of the relative ease with which they can be quantitatively manipulated (2). For example the synthesis of RNA (3–5), total cellular protein (3), histones (6–8), collagen (9), immunoglobulins (10), and specific unidentified proteins (10 *a*) have been shown, in a number of different cultured cell systems, to occur at rates which depend on the cells' age. The replication of the centrioles (3, 11), and large increases in the activity—and presumably synthesis—of a number of enzymes (12–18 *a*), occur at particular times in the cell cycle, as does susceptibility to hormonal regulation of enzyme activity (19). Other physiological parameters also show age-dependent fluctuations; for example the responses of cells to a variety of chemical and physical agents (see references 20, 21 for references), including ionizing radiations (22–24), depend on their position in the generation cycle.

These variations, which must depend at least indirectly on the operation of one or another cellular clock, might be expected to be accompanied by variations in flux and/or content of nonmacromolecular cellular metabolites. However, information concerning these is meager; other than measurements of the rates of incorporation of precursors of nucleic acids and proteins, the only report on this subject of which we are aware concerns a discontinuity in the rate of Na^+ and K^+ transport at the beginning of the S phase in mouse leukemic cells (25).

The possibility that the sulfhydryl content of cells may vary cyclically has been recognized for some time. In 1931 Rapkine (26) described a sequence of changes, related to cell division, in the sulfhydryl content of a protein isolated from sea urchin eggs. His results have been confirmed and extended by several workers (27, 28), and have been related to the formation of the mitotic apparatus. However, no reports have hitherto been published on the age-specific sulfhydryl content of unperturbed, synchronous mammalian cells (other than a comparison of mitotic with interphase HeLa cells; reference 29). The present experiments, which were carried out with synchronous cultures obtained by a selection method (30), show that characteristic fluctuations occur in the content of protein- and nonprotein-bound sulfhydryl groups, of protein-bound disulfide linkages, and of disulfide linkages between proteins and small molecules. Data are also presented concerning the changes that occur as an asynchronous culture progresses from lag phase through the exponential phase to the stationary phase of growth. Finally, since sulfhydryl groups are found largely as constituents of proteins, data are reported on the age-specific protein content of these cells. The results can be interpreted partly in terms of the postulated role of sulfhydryl groups in the formation of biopolymers containing disulfide linkages (27). These changes will be discussed in relation to the radiation sensitivity of the cells in a subsequent communication.[1]

[1] Mauro, F., and L. J. Tolmach. 1969. Data in preparation.

MATERIALS AND METHODS

Abbreviations

PSH	protein-bound sulfhydryl groups
PSSP	protein-bound disulfide groups
PSSQ	disulfide groups linking a protein with a nonprotein
QSH	nonprotein-bound sulfhydryl groups
QSSQ	nonprotein-bound disulfide groups
—SH	sulfhydryl [groups]
—SS—	disulfide [bridges]
—SH & —SS—	total sulfhydryl plus disulfide groups
PCA	perchloric acid

Cell Cultures

HeLa S3 cells were grown in medium N16 supplemented with a 1:2 mixture of horse and human serum to give a final serum concentration of 30% (31). The mean generation time was about 22 hr; the plating efficiency was between 75 and 100%. Asynchronous cells in the exponential and stationary phases of growth were obtained from plastic Petri dish (87 mm I.D.) cultures inoculated with 1.5×10^5 stationary phase cells in 13 ml of medium; after 2 days the cells were in the exponential phase and at a concentration of about 5×10^6 cells/dish; after 4 days the cells reached the stationary phase at a concentration of $1.5–2.0 \times 10^6$ cells/dish. Cells were counted after trypsinization, using a Coulter electronic cell counter. Synchronous populations were obtained by mitotic cell selection (30). Suitable numbers of harvested cells were plated in Petri dishes and incubated under standard conditions (37°C, 4.5% CO_2 in humidified air) until analyzed. Cell division in synchronized cultures was monitored by repeated microscopic observations of representative fields (32). Purification of G_2 populations was carried out in certain experiments, as described in the Results (Figs. 6 and 7).

Washing of Cells

Upon addition of 0.2 N perchloric acid (PCA) to cells from which the medium had been decanted, copious quantities of a flocculent precipitate were formed. If included in the material analyzed for protein or protein-bound sulfhydryl groups, erroneously large values were obtained. This precipitate presumably consisted of proteins from the serum component of the medium. Some of these appeared to be tightly bound to the cell surface: repeated washing of the cells with Puck's Saline D1 (Grand Island Biological Company, Grand Island, N. Y.) progressively reduced the volume of precipitate, but the analytical values obtained (in the absence of PCA treatment) did not reach constant levels even after 6 rinses. However, when the cells were treated with PCA after two or more saline rinses (procedure A below), constant analytical values were obtained; the acid treatment apparently removed the residual material. Similarly, treatment with trypsin (procedure B below) for at least 2 min after two saline washes also appeared to remove the residual material, as the analytical values did not decrease with further pre-trypsin washing, and agreed with those obtained after the PCA treatment (see Results). This agreement strongly suggests that the material removed by the saline, PCA, and trypsin treatments is indeed extracellular, and that it is completely removed by these treatments. It may be noted, furthermore, that these procedures do not appear to result in the loss of intracellular substances: the same content of nonprotein sulfhydryl

groups was measured in cells washed one to six times, with or without subsequent treatment with PCA or trypsin.

Determination of Protein

Protein content was measured by the Oyama and Eagle modification (33) of the Lowry procedure (34), using bovine serum albumin as a standard. Cells were rinsed twice with Saline D1 and fixed directly in the plastic dishes with 0.2 N PCA for at least 1 hr at about 4°C. The dishes were then rinsed once with cold distilled water and twice with cold absolute ethanol, and dried at room temperature. Finally, the cells were dissolved in 1 ml of the alkaline solution prescribed in the analytical procedure, and the protein content was determined.

Tracer Incorporation Measurements

The rate of DNA synthesis was monitored by adding small volumes (20 μl) of thymidine-^{14}C (0.05 μc/dish; 60 c/mole) to 1 ml cultures (in wax ringed 34 mm dishes; see 30) for 20 min. To measure the incorporated radioactivity, the cultures were rinsed, fixed, and dried as above, and the dish bottoms were punched out (30) and counted in a low background gas-flow Geiger counter. The rates of incorporation of the labeled amino acids valine-^{14}C and arginine-^{14}C (10 μl of stock solution; 6 μc/dish; 150 c/mole were determined in similar fashion.

Fractionation of Cells for Determination of Protein-Bound and Nonprotein Sulfhydryl and Disulfide Groups

Fig. 1 is a schematic outline of the two procedures (designated A and B) employed to prepare cell fractions for titration. The use of two different methods served to check the adequacy of the separations; in particular it was necessary to demonstrate that separation of protein-bound from nonprotein species was effected by centrifugation of the unfragmented, acid-fixed cells in procedure A.

Procedure A involved fixing washed cells with 0.2 N PCA for at least 1 hr at about 4°C, detaching them from the dish with a few drops of 0.1 N NaOH, neutralizing the suspension within 1 min with a large volume of cold acetate buffer, and centrifuging it at about 7000 *g* for 15 min to separate protein-bound from nonprotein groups. Analysis of the cell supernatant for protein showed that 98.6% of the total was sedimented. (99.7% of the total was sedimented if the suspension was reacidified with PCA before centrifugation.) Apparently, only a negligible amount of protein was solubilized by the short treatment with NaOH and suspension in buffer; however, if the 1 min treatment was prolonged to 15 min, 16% of the protein appeared in the supernatant. Care was exercised to restrict the NaOH treatment to 1 min. A portion of the supernatant was titrated to determine the concentration of nonprotein-bound sulfhydryl groups (QSH). To measure the nonprotein-bound disulfide (QSSQ) content of the same supernatant, an aliquot was treated with 20 mg/ml sodium borohydride for 30 min in an agitated waterbath at 40°C (35), prior to titration. This reagent has been demonstrated to reduce disulfide bonds (36, 37). The QSSQ content was obtained from the measured value by substracting that for QSH.

Preliminary test of the availability of titratable groups in protein that had been precipitated with PCA and resuspended in acetate buffer was carried out with bovine serum albumin. Identical values (which agreed with established ones) were obtained whether samples were titrated directly or were treated with PCA and resuspended in buffer before titration. Hence, the pellet from the cell centrifugation was suspended in acetate buffer and examined for its protein-bound sulfhydryl (PSH) and disulfide (PSSP) content. Direct titration of this material

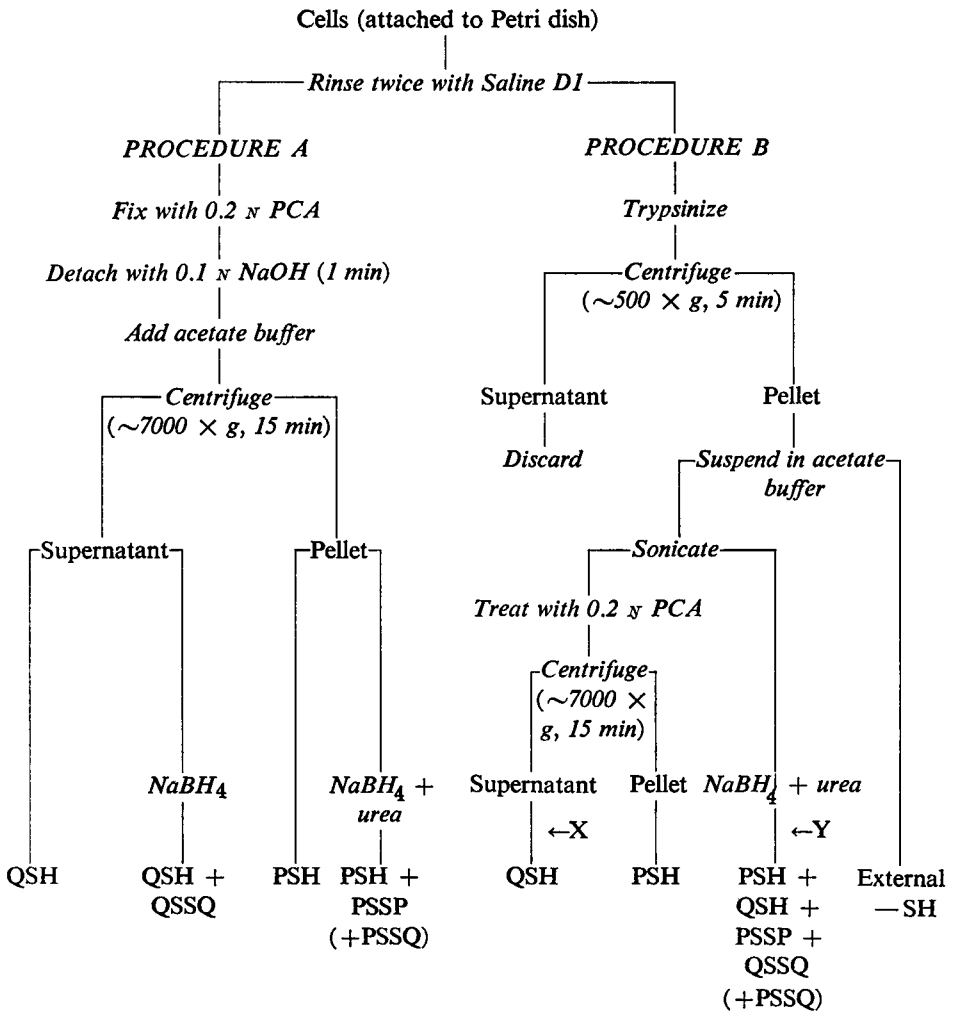


FIGURE 1 The two procedures used to prepare cell fractions for determination of protein-bound and nonprotein sulfhydryl and disulfide groups.

yielded a value for PSH. (Treatment of the pellet with 8 M urea to unfold proteins increased the measured content of PSH by only 5%, irrespective of the age of the cells. Urea treatment at this point was accordingly omitted.) Pretreatment of the pellet as before with $NaBH_4$, in the presence of 8 M urea, yielded a value for PSH + PSSP. By subtracting the value for PSH, the PSSP content was obtained. (In contrast with the PSH determination, reduction of —SS— by $NaBH_4$ without urea treatment yielded only 70% of the titratable groups measured after reduction in the presence of urea.) In this procedure, any mixed disulfides between proteins and small molecules (PSSQ) that may be present (35) are measured together with PSSP, as in the results presented in Table II and in Figs. 3 and 5.

In procedure B the washed cells were dislodged from the dish by at least 2 min treatment with 0.03% trypsin (prepared in acetate buffer instead of Saline D1; preliminary experiment

showed that this modification had no effect on the action of the enzyme), sedimented by centrifugation at 500 *g* for 5 min, and resuspended in cold acetate buffer. (More extensive washing to remove possible residual trypsin did not affect the results.) These cells were either directly analyzed or were first sonicated (3 min at maximum output of a Branson LS-75 Sonifier; the temperature did not rise above 37°C). Titration of the nonsonicated material yielded a value taken to represent the concentration of —SH on the outer surface of the cell. The sonicated material, after treatment with 0.2 N PCA and centrifugation at 7000 *g* for 15 min, yielded values for QSH (supernatant) and PSH (pellet). The same material, after pretreatment with $NaBH_4$ and urea, provided a value for the total cellular —SH and —SS— groups, from which the content of QSSQ + PSSP was determined by difference. (In separate tests, it was found that the use of trypsin to dislodge cells from the dish yielded values for the total sulfhydryl plus disulfide groups (-SH & -SS-) and for QSH that were the same as those measured in cells dislodged with a "rubber policeman." Since trypsinization resulted in no loss of either thiols or SH-containing proteins, and gave a higher yield of cells, its use was adopted.) As in procedure A, the value for PSSP includes that for PSSQ. In order to measure the PSSQ content of cells, procedure B was modified by introduction of a reduction step ($NaBH_4$) at point *X* (Fig. 1), and a precipitation (PCA) and centrifugation (7000 *g*, 15 min) step at point *Y*. In this way the QSH arising from PSSQ could be distinguished from both those arising from QSSQ and those present as such in the cell.

Titration of Sulfhydryl Groups

Numerous methods have been described for the quantitative estimation of sulfhydryl groups (38). For the present work a highly sensitive method was necessary because of the relatively small numbers of cells available. Accordingly, the spectrophotometric titration devised by Klotz and Carver (39), which can be carried out conveniently with as few as 300 cells, was adopted; generally at least 500 cells were analyzed. The method is based on the fact that mersalyl acid (MA; also called salyrganic acid), a mercurial, reacts stoichiometrically with —SH groups before reacting with pyridine-2-azo-*p*-dimethylaniline (PADA) to produce a colored product; the PADA serves as an indicator that reaction of the —SH groups is complete. The titration procedure was carried out as follows, using 0.1 M acetate buffer at pH 5.8 as solvent for all reagents (which were obtained commercially). Solutions of MA were prepared at appropriate concentrations in the range 10^{-3}–10^{-6} M (5×10^{-3} M NaCl was added to solubilize the mercurial). Nine volumes of PADA at a concentration of 8×10^{-5} M were mixed with one volume of the solution whose —SH content was to be determined, MA was added in increments, and the absorbance, measured with a Beckman DU spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.) at 550 nm in a 1 or 3 ml cuvette with 1 cm optical path, was recorded. Near and beyond the equivalence point, increments of 0.02 ml of MA were added. The —SH contents of standard solutions of two thiols, mercaptosuccinic acid and glutathione, were first titrated to check the accuracy of the determination.

Remarks Concerning Analytical Methods

If any oxidation of —SH groups by air occurred during the analytical procedures, it was limited, since large amounts of PSH and QSH but only small amounts of QSSQ were found (see Results), and must have taken place within the brief period (as short as 10 min) preceding titration, as the measured values of total —SH content were constant for at least 2 hr.

Values for the disulfide content are given in terms of —SH, i.e., as twice the —SS— concentration. The measured values of —SH and —SS— groups in synchronous populations

TABLE I

SULFHYDRYL AND DISULFIDE CONTENTS OF EXPONENTIALLY* GROWING HELA CELLS AS DETERMINED BY TWO FRACTIONATION PROCEDURES

Fractionation procedure‡	CONSTITUENTS§		Total —SH & —SS—
	QSH + PSH	QSSQ + PSSP	
	moles per cell ($\times 10^{15}$)		
A	24.4 ‖	5.7‖	30.1
B¶	24.6	6.1	30.7

* 48-hr cultures.

‡ See Fig. 1, and text.

§ Disulfide contents arereported as —SH. See text for abbreviations.

‖ See Table II for individual values.

¶ The high speed centrifugation step was omitted.

are presented in terms of one cell of standard protein content; this normalization is discussed in the Results.

The results reported refer to individual experiments. Most determinations were repeated at least four times; in general, replicate experiments using a given procedure yielded values within 5% of each other. The results obtained for cultures fractionated by the two procedures are in reasonably good agreement, as is evident from the comparison shown in Table I (the high speed centrifugation step was omitted from procedure B) and from the data presented in Figs. 4A and 5. Furthermore, in certain experiments utilizing procedure B, the sum of the individual components could be compared with the value for —SH & —SS— measured directly; agreement was within 7%.

RESULTS

Changes during Asychronous Growth of a Culture

Before measuring the sulfhydryl and disulfide contents as a function of age in the cell cycle, asynchronous cultures in different phases of growth were examined. The cultures were inoculated with cells from a trypsinized population that had grown to the stationary phase; they were examined at intervals during the lag, exponential, and stationary phases (Fig. 2 C). In these experiments the —SH and —SS— contents were determined by procedure A only (see Materials and Methods), except for the 48-hr cultures (middle of the exponential phase) which were also partially fractionated by procedure B (Table I).

The total —SH & —SS— content as a function of culture age is shown in Fig. 2 B. An increase of somewhat more than a factor of 2 occurs during the lag phase; thereafter, the concentration drops gradually during the exponential phase, returning to the initial value as the cells enter the stationary phase. The content in the middle of the exponential phase is about 28×10^{-15} moles/cell. Because a large

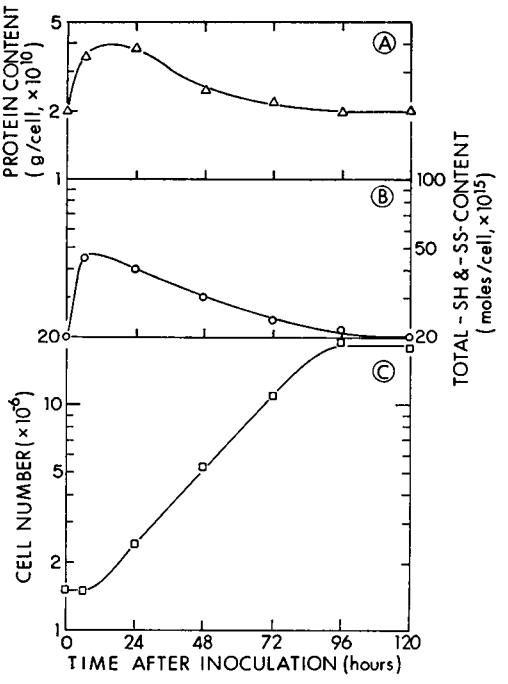


FIGURE 2 Cellular protein content (panel A), total cellular —SH & —SS— content (panel B), and cell number (panel C) during asynchronous growth of a culture inoculated with stationary phase cells at zero time. The data for —SS— are in terms of —SH. Semilogarithmic coordinates are used in panels A and B arbitrarily.

fraction of the —SH & —SS— content occurs as protein-bound material (see below), these changes would be expected to follow those of the protein content of the cell as the cultures grow. That this is a case is evident from the data in Fig. 2 A in which the protein concentration is shown as a function of time. The protein content of the cell undergoes a rapid increase during the lag phase, from 2×10^{-10} g at inoculation to about twice this value after 1 day. During the exponential phase it slowly drops, returning to the initial level by 4 days when the cultures reach the stationary phase. This result is similar to that previously reported by Salzman (40) for HeLa cells; it is also consistent with that found in this laboratory several years ago (E. Diamant, unpublished data). It may be noted that the average volume of HeLa cells decreases in a similar fashion during the growth of a culture, reaching a minimal value in stationary phase (unpublished data). Within the limits of error of the experimental measurements, the changes in protein content are identical with those in —SH & —SS— content. If the —SH & —SS— values are normalized to a constant protein content per cell, the data lie on a horizontal line at 1.1×10^{-6} moles of —SH & —SS—/gram of protein. This value for HeLa cells is between 40 and 55 % of that reported by Modig (35) for Ehrlich ascites tumor cells; it is also of the same order as that reported for other cells (41). Assuming an average protein molecular weight of 50,000, it corresponds to about 2 —SH/molecule.

Table II lists the cellular contents of protein-bound and nonprotein —SH and —SS— groups. The four measured components occur in very unequal amounts;

TABLE II

SULFHYDRYL AND DISULFIDE CONTENTS OF HELA CELLS AT SEQUENTIAL TIMES DURING GROWTH OF AN ASYNCHRONOUS CULTURE*

Time after inoculation of culture	CONSTITUENT‡				Total —SH & —SS—
	QSH	QSSQ§	PSH	PSSP§‖	
hr	*moles per cell* ($\times 10^{15}$)				
0	0.4	0.4	15.0	4.3	20.1
6	4.1	0.4	32.6	8.3	45.4
24	3.8	0.9	29.3	6.4	40.4
48	2.9	0.5	21.5	5.2	30.1
72	2.4	0.6	16.4	4.8	24.2
96	0.8	0.7	16.5	4.1	22.1
120	0.4	0.5	15.2	4.1	20.2

* Cells were fractionated by Procedure A (see Fig. 1, and Materials and Methods).
‡ See Materials and Methods for abbreviations.
§ Disulfide contents are reported as —SH.
‖ Includes PSSQ.

about 90% of the groups are associated with protein, and of these, about 80% are in the —SH form. While there are systematic changes in the amounts of the protein-bound components, they fluctuate over only a factor of 2, and in parallel with the protein. The QSSQ content, which remains below 1×10^{-15} moles/cell at all times, varies in a similar fashion. In contrast the QSH content fluctuates widely: it is strongly correlated with the growth stage of the culture, increasing from 0.4 to 4.1×10^{-15} moles/cell during lag phase, dropping slowly, in parallel with the protein content, during exponential growth, and then falling rapidly as the cells enter the stationary phase. It falls further while the culture is maintained for 1 day in the stationary phase; whether the decrease continues with longer incubation has not been determined.

The PSH content found here for exponentially growing cultures of HeLa S3 cells, 21.5×10^{-15} moles/cell at 48 hr after inoculation, is of the same order as that reported for Ehrlich ascites tumor cells (e.g. 37) but is 4- to 5-fold lower than that found for human kidney cells (42). The QSH content, 2.9×10^{-15} moles/cell, is also of the same order as that reported for Ehrlich cells (35, 43–45), but is about 20% that of Chinese hamster (46) and human kidney cells (42), and only one-tenth that reported for HeLa cells by Harris et al. (46). Some of these apparent discrepancies may constitute true differences among cell types, perhaps reflecting, in part, variation in cell size. (This matter is discussed further in the following section.) It is of interest that in all cases reported, the ratio of the PSH to the QSH content lies between 5 and 10.

The fractions of the total —SH & —SS— complement of the cell represented

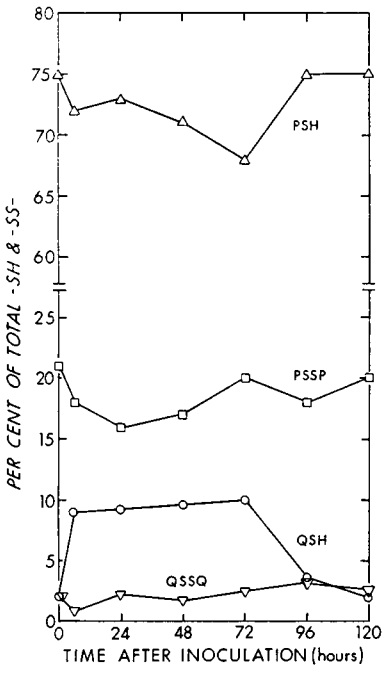


FIGURE 3 Relative cellular content of protein sulfhydryl (PSH), protein disulfide (PSSP), nonprotein sulfhydryl (QSH), and nonprotein disulfide (QSSQ) groups during asynchronous growth of a culture. The data for the disulfides are in terms of —SH. Those for PSSP include mixed disulfides (PSSQ).

by each of the four measured components are shown as a function of time in Fig. 3. When compared on this basis, the contents of PSH and PSSP show only small changes during growth of the culture from inoculation to well into the stationary phase; protein-bound species together fluctuate between about 96% of the total in the lag and stationary phases, and about 88% during exponential growth. Since this method of data comparison automatically takes into account the changes in protein content, the observation that the fractional contents of PSH and PSSP are relatively constant is not surprising. The fractional QSH content, on the other hand, undergoes a 4- to 5-fold increase during lag phase, remains at the higher level for 3 days, and then declines to the initial level during the next 2 days as the cultures enter the stationary phase. A similar though somewhat smaller difference in QSH content of exponential and stationary phase Chinese hamster cells has recently been reported (46).

The disappearance of the nearly 2-fold decrease in QSH content between 6 and 72 hr (Table II) when the data are expressed on a percentage basis (Fig. 3) suggests that the QSH concentration might be related to the protein content. However, the 5-fold changes in the fractional QSH content during lag phase, and particularly during stationary phase (from 72 to 120 hr) when the amount of protein remains constant, indicate that this relation merely reflects a correlation between cell size and protein content. In the next section it will be shown that the QSH concentra-

tion is strongly dependent on the stage of the cell cycle. The possibility of interconversion of the various groups is also considered.

Measurement of the cellular content of mixed disulfides, PSSQ, was carried out on 24 hr random cultures, using the modification of procedure B outlined in Materials and Methods. A value of 1.4×10^{-15} moles/cell was obtained. Comparison with the value of 6.4×10^{-15} moles/cell (as —SH) for total protein disulfide groups in 24 hr cultures (Table II) indicates that 44% of the latter are mixed disulfides. This value is in reasonable agreement with that of 30% reported by Modig for Ehrlich ascites tumor cells (35).

Changes during the Cell Cycle

Protein-bound and nonprotein sulfhydryl and disulfide groups were determined at frequent intervals during the generation cycle of synchronously growing cells. Data were obtained over a 24 hr period (one cycle) for cells harvested by mitotic selection from exponential phase cultures (46–50 hr after inoculation). The progress of these cultures through the cell cycle is well established (30); as shown in Fig. 4 D, DNA synthesis (measured by incorporation of thymidine-^{14}C into the culture) begins at about 7 hr after cell collection, and its rate peaks at about 13 hr. The diagram at the bottom of Fig. 4 shows the approximate age composition of the population during the experiment.

Because it was found with asynchronous cultures that a large fraction of the

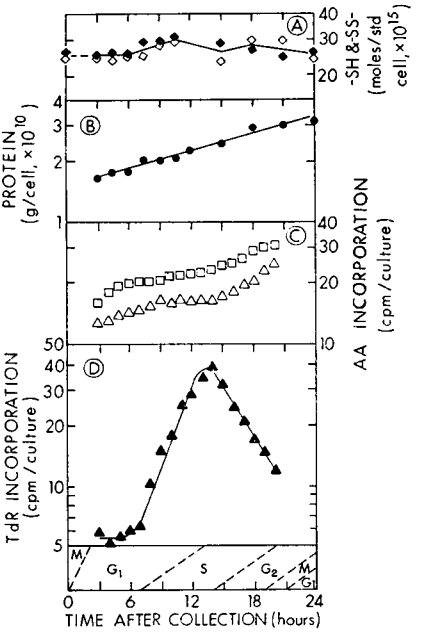


FIGURE 4 Panel A shows the total cellular content of —SH & —SS— groups as a function of the age of a synchronous culture whose approximate age composition is shown by the diagram at the bottom of the figure. The data are expressed in terms of a standard cell containing 2.5×10^{-10} g of protein. Open symbols refer to determinations on material fractionated by procedure A, and closed symbols by procedure B (Fig. 1). The actual cellular protein content is shown in panel B. Panel C shows the rate of incorporation of two labeled amino acids (AA), arginine-^{14}C (squares) and valine-^{14}C (triangles), into acid-soluble cell material as a function of cell age, and panel D, that of ^{14}C-labeled thymidine (TdR) into DNA. Semilogarithmic coordinates are used for convenience. Note that the values are not corrected for the increase in cell number which begins at about 19 hr.

cellular —SH & —SS— content is protein bound, protein was determined in the synchronously growing cells also. Fig. 4 B shows that the protein content increases from about 1.5 to 3.1 $\times$ 10^{-10} g/cell during a cycle. (The value of 2.5 $\times$ 10^{-10} g/cell measured for 48 hr asynchronous cultures [Fig. 2] is somewhat higher than would be expected for a growing population distributed around the cell cycle according to random division.) The data indicate that there is a continuous increase in protein, but do not permit discrimination between various possible modes of accretion. (The data are plotted on semilogarithmic coordinates for convenience only.) However, indication that protein might increase exponentially is afforded by measurements of the rates of incorporation of two labeled amino acids, arginine-^{14}C and valine-^{14}C, into acid-insoluble cell material. Fig. 4 C indicates that both rates approximately double during the cycle, suggesting that the rate per unit protein remains constant. The rates do not appear to increase continuously; both show apparent plateaux in late G_1 or early S, the plateau with arginine perhaps occurring somewhat earlier than that with valine. However, in view of the possibility that the measurements reflect changes in the size of precursor pools during the cycle, in addition to changes in rates of protein synthesis, these findings must be interpreted with extreme caution.

The cellular —SH & —SS— content, normalized (see next paragraph) to a standard protein content of 2.5 $\times$ 10^{-10} g (Figs. 2, 4 B), shows little variation during the cell cycle (Fig. 4 A). It rises from about 25 to about 30 $\times$ 10^{-15} moles during late G_1, and then gradually falls to the initial value during the remainder of the cycle. These changes are probably somewhat more accurately depicted by the data obtained by procedure B (solid symbols) than by procedure A (open symbols), as the former yields direct measurements of the total content of the 4 species. The values (without normalization) are somewhat lower than would be expected from that for 48 hr random cells (30.1 $\times$ 10^{-15} moles; Table II). The external —SH concentration, determined in procedure B, also changes in parallel with the protein content during the cycle; there are about 0.5 $\times$ 10^{-15} moles/standard cell, corresponding to 2% of the —SH & —SS— content.

Fig. 5 shows the intracellular concentrations of the four individual groups during the cycle. Cells were fractionated by both procedure A (open symbols) and B (closed symbols). The values for PSSP in procedure B were obtained from the difference between the values for the sonicated material with and without treatment with $NaBH_4$ + urea, subtracting the average value of QSSQ measured in procedure A. (It should be noted that the values for PSSP include those for PSSQ; see below.) Again, the data are expressed in terms of a standard cell containing 2.5 $\times$ 10^{-10} g of protein, the value found for asynchronous populations at 48 hr (which corresponds to the value measured at 16 hr after mitosis in synchronous cultures). This has been done in order to eliminate the changes in PSH and PSSP (which together constitute about 90% of the total —SH & —SS—) that would be expected from

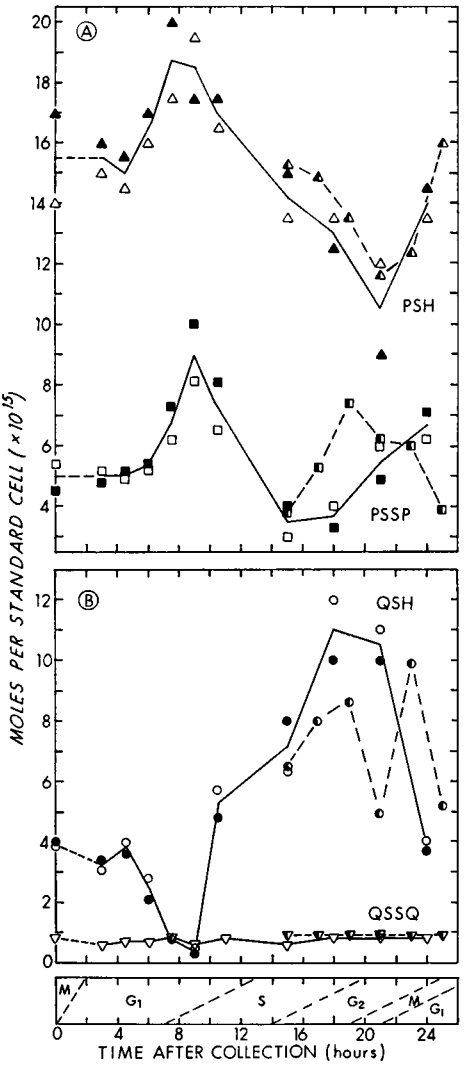


FIGURE 5 Cellular content of protein sulfhydryl (PHS) and disulfide (PSSP) groups (panel A) and of nonprotein sulfhydryl (QSH) and disulfide (QSSQ) groups (panel B) as a function of cell age during synchronous growth. (The approximate age composition of the population is shown in the diagram at the bottom.) All the values shown by the open and closed symbols, except those for mitotic (0 hr) cells, were obtained in a single experiment, the open symbols referring to procedure A, and the closed to procedure B (Fig. 1). The continuous lines connect the means. The values are expressed in terms of a standard cell containing 2.5×10^{-10} g of protein; those for disulfide groups are given as —SH; those for PSSP include mixed disulfides (PSSQ). The half-closed symbols (dashed lines) show the results obtained with purified populations (see text), using procedure B.

increase in protein content alone. (The values for the nonprotein species are similarly normalized, both for consistency and because they might be expected to increase with increasing cell mass or volume, and hence protein content. It may be noted that this normalization changes the shape of only the PSH curve.) All of the foregoing data in Fig. 5, except those for the collected mitotic (zero-hour) cells, were obtained in a single experiment. Although the reproducibility of replicate experiments utilizing a given procedure was generally within 5%, there are frequent instances of greater than 5% discrepancy between values obtained by the two procedures. It is not clear to what these should be attributed; however, because there

is a tendency for the values obtained at a given time by one procedure to be higher than those obtained by the other procedure, it is likely that inaccuracies in the apportionment of the samples, rather than in the fractionation procedures or the determination of the equivalence points, generally dominated the analyses. Inasmuch as there is no basis on which to choose between them, the two values have been averaged at each time; the continuous line segments join these averages.

The QSSQ content shows only small, if any, variations during the cycle, remaining at the relatively very low value of about 0.7×10^{-15} moles/standard cell (that is, the absolute QSSQ content increases steadily to twice its initial value during the cycle), in agreement with the value of 0.5×10^{-15} moles measured in 48-hr random cultures (Table II). The three remaining species, in contrast, undergo large fluctuations. Of these QSH shows by far the largest percentage change during the cell cycle. Its concentration falls from about 4×10^{-15} moles/standard cell (or 2.4×10^{-15} moles without normalization) in mitosis to about one-tenth this value during the latter part of G_1. It rises abruptly as the cells enter S and continues to rise more slowly during much of the S phase, reaching 11×10^{-15} moles/standard cell (or 12×10^{-15} moles without normalization) at 18 hr, which is about three times the mitotic value. It appears to return to the initial value by 24 hr. Thus, there is about a 30-fold overall fluctuation in normalized QSH content during the cycle; of course, the absolute value fluctuates even more. The value of 2.9×10^{-15} moles/standard cell measured in 48-hr random cultures (Table II) is in reasonable agreement with these measurements.

These age-dependent fluctuations in QSH content are compatible with the observation of Harris et al. (46) that HeLa S3 cells (synchronized by a chemical blocking method) have a higher QSH content in late S phase than in late G_1. However, the values reported by those workers for late S cells are about five times those found here. Furthermore, their reported ratio of the late-S to late-G_1 content is smaller. In addition presumably different patterns of variation in QSH and PSH content have been reported for human kidney cells (42). These divergent findings may in part reflect characteristic properties of different cells; however, the use of different methods of synchronization might be an important cause of the discrepancies.

The fluctuations in the intracellular content of QSH are mirrored by the changes in PSH, and to a lesser extent in PSSP. While the latter two components show both maxima and minima that are apparently a few hours out of phase with each other, the general trends of both of them are opposite to those of QSH. The magnitudes of these changes are such that the fluctuations in QSH are approximately compensated (hence the small changes in —SH & —SS— content), PSH showing greater fluctuation. The PSH content increases from about 15 to about 19×10^{-15} moles/standard cell (from 10 to 15×10^{-15} moles without normalization) during late G_1. It then drops steadily during the remainder of the cycle, to about 10×10^{-15} moles/

standard cell (12×10^{-15} moles without normalization). The concentration of PSSP also increases during late G_1, from 5 to about 9×10^{-15} moles/standard cell (from 3.5 to 7×10^{-15} moles without normalization), and then drops more rapidly than does that of PSH, reaching a minimum of about 3.5×10^{-15} moles/standard cell (3×10^{-15} moles without normalization) by 15 hr; it apparently rises during G_2. (Resolution of the measured values of PSSP into values for PSSP and PSSQ is discussed below.) The value for PSSP in randomly growing cells (Table II) is in reasonable agreement with the value (without normalization) measured in the synchronous populations. However, that for PSH is higher than any of the values measured in synchronous populations.

Because the degree of synchronization decays during the cell cycle so that measurements made in its latter portion yield values for mixtures of cells in late S and G_2, together with cells in M and early G_1, additional experiments were carried out with partially purified populations in which the cells examined were confined to one generation. Purification was effected by periodically removing cells which entered mitosis and rounded up, using for this purpose the usual mitotic cell selection procedure (30) at half-hour intervals; Fig. 6 shows the fraction of cells remaining attached to the dish at each time of analysis. In this way the population examined consisted only of G_2 and some S phase cells, and was not contaminated by mitotic or G_1 cells. The results of the analyses, for which procedure B was used, are shown in Fig. 5 (half-closed symbols, dashed lines) for ease of comparison with those for the crude populations. The increased resolution reveals additional fine structure; in particular a minimum appears in the curve for QSH at 21 hr, and the final drop in the QSH content is delayed, presumably because there has been selection for the more slowly progressing portion of the population. In addition the rise in PSSP begins about 3 hr earlier in the purified population, and is followed by a return to close to the mitotic level by the end of the experiment. Although the maximum occurs 2 hr earlier than the minimum in the QSH curve, the peak is broad, and spans the region of the trough in the QSH curve. The PSH and QSSQ values are close to those

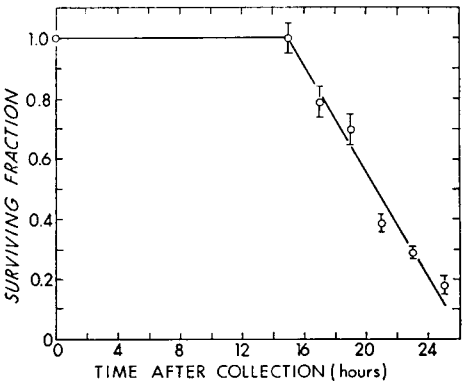


FIGURE 6 Fraction of total cells remaining attached to Petri dishes (Surviving Fraction) as a function of time, on periodic removal of mitotic cells. A group of dishes was inoculated with mitotic cells at zero time. Rinsing of dishes to remove rounded cells was first carried out at 15 hr, and was repeated at half-hour intervals; the population was thereby restricted to the first generation. A separate culture was trypsinized, counted, and fractionated by procedure B (see Fig. 1) at the indicated times (see Figs. 5, 7). The bars indicate the standard errors of the means.

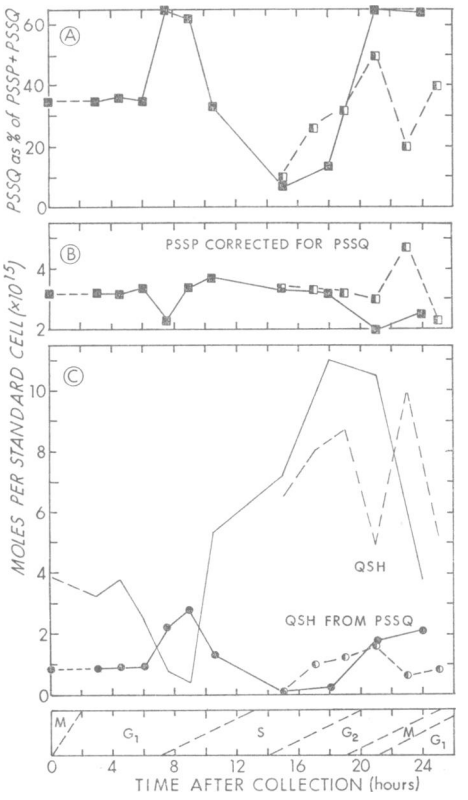


FIGURE 7 The cellular content of mixed disulfide groups (PSSQ), determined by procedure B and expressed in terms of a standard cell containing 2.5×10^{-10} g of protein, is shown by the symbols in panel C as a function of cell age during synchronous growth. The lines without symbols show the data for nonprotein sulhydryl groups (QSH) from Fig. 5 B, for comparison. Panel B shows the PSSP content (Fig. 5 A) after subtraction of the PSSQ component. Panel A shows the percentage of the total protein disulfide groups arising from PSSQ. The half-closed symbols (dashed lines) show the results obtained with purified populations. The approximate age composition is shown by the diagram at the bottom.

found in the crude populations. (Purification was also carried out by adding vinblastine sulfate to synchronous cultures at 15 hr, before any cells had reached mitosis, and removing the trapped mitotic cells immediately before analysis at subsequent times (4, 47). While the results were qualitatively similar to those obtained by the purely mechanical procedure described above, they are of dubious quantitative significance because vinblastine interferes with mitotic spindle formation, and might drastically perturb the formation of intermolecular disulfide bridges: see Discussion.)

A further series of experiments was carried out in order to measure changes in the cellular content of mixed disulfides, PSSQ, during the cell cycle. Fig. 7 C shows that the PSSQ content (measured as QSH) rises from 0.9 to about 2.8×10^{-15} moles/standard cell at the G_1/S transition, and then drops nearly to zero before rising again later in the cycle (closed circles). The initial peak occurs at the time of the minimum in the QSH curve (reproduced from Fig. 5 for comparison). In purified populations (half-closed circles) the second rise occurs earlier than in the crude population, and from 19 hr on, the changes roughly mirror those in QSH, but are of much smaller magnitude.

Determination of the PSSQ content permitted its subtraction from that for PSSP previously measured (Fig. 5). When so corrected, the changes in PSSP content (mean values) are largely damped out (Fig. 7 B), although a small minimum occurs at 7.5 hr, and a second drop occurs late in the cycle (closed squares). The latter, however, is largely eliminated and replaced by a modest peak at 23 hr in purified populations (half-closed squares), which coincides with the minimum in the PSSQ curve. It should be noted that there is considerable uncertainty in the derived values, as there were sizable (20%) discrepancies between certain of the PSSP values determined by procedures A and B. Fig. 7 A depicts the fraction of the total disulfide content that occurs as mixed disulfides. It is seen that this fluctuates widely, reaching 65% at the G_1/S transition and in G_2 (50% in G_2 in purified populations; half-closed symbols), and falling to less than 10% in the middle of the S phase.

DISCUSSION

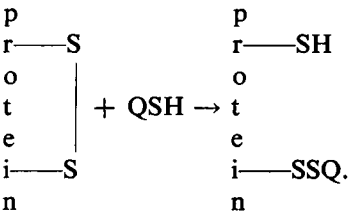
The results of the present work show that the total content of sulfhydryl and disulfide groups in HeLa cells, normalized to a standard protein content, does not vary significantly during growth of asynchronous cultures (Fig. 2). This behavior reflects the fact that about 90% of these groups occur bound to proteins, and that the cellular content of the protein-bound species fluctuates in close correspondence with the protein content as cultures pass from lag to stationary phase (Table II). The concentration of QSH, in contrast, shows much larger fluctuations, decreasing markedly as cultures enter the stationary phase where it reaches a level as low as that achieved by synchronous populations at the G_1/S transition (Fig. 5); that is, the QSH content is strongly correlated with cell proliferation. The constancy of the fractional QSH content during the exponential phase, together with the large changes that occur during the lag and stationary phases (Fig. 3), suggest that the concentration of QSH might be a useful indicator of the growth phase of a culture.

The fluctuations in the concentrations of PSH and PSSP are much larger during a single replication cycle in synchronous cultures (Fig. 5) than they are during the growth of asynchronous cultures. The content of PSH drops from 65% of the —SH & —SS— at 9 hr (G_1/S transition) to 45% at 18 hr (S/G_2 transition), and that of PSSP from 31 to 13%. Much of the latter change arises from changes in the concentration of PSSQ. But again the QSH content shows the largest changes; it increases from 1 to 38% of the total during this interval. It may be noted in this connection that the QSH content in stationary phase cultures (Table II) is the same as that of late G_1 cells.

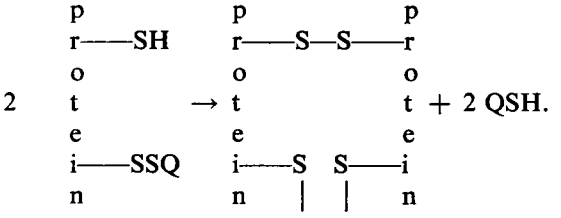
The extremely low QSH levels at the G_1/S transition are of interest also in view of the changes in a number of cellular parameters which occur at that stage of the cell cycle. For example histone synthesis (6–8) and centriolar replication (3, 11) begin at that time, the rate of synthesis of RNA starts a 2-fold increase (3–5), the rate of protein synthesis increases (3), the flux of Na^+ and K^+ begins to double

(21), and the activities of a number of enzymes associated with DNA synthesis change (e.g. 12–15). In addition cultured cells show a markedly greater response to exposure to certain toxic agents, including X-rays, at the G_1/S transition (21–24, 48–50). Thiols might be involved in any of these phenomena. For example they are known to alter the response of biological systems to ionizing radiations (51), and the age-specific content of QSH is very similar to the age-response of HeLa cells to X-rays (cf. Fig. 5 with Fig. 10 of reference 48). However, as will be discussed in a subsequent paper,[1] the situation is far more complicated than this simple correlation implies.

It appears, then, that the present findings may be pertinent to several areas of cell biology. Sulfhydryl and disulfide groups play a number of different roles in the metabolism of the cell—as constituents of cofactors and prosthetic groups of enzymes, and as structural and/or functional elements in the formation of certain proteins—and the age-dependent fluctuations described here might be associated with any of these functions. Because the last was felt possibly to be the one most likely to be related to the large changes observed, it was examined in detail. It has been proposed by Mazia that the assembly of protein subunits into the mitotic apparatus involves a transient formation of PSH groups which act as intermediates in the conversion of intramolecular to intermolecular disulfide linkages (27). The intramolecular —SS— groups are presumed to react with thiols, such as glutathione, according to the reaction



The intermediates are supposed to subsequently combine, with the release of the thiols:



The data presented in Figs. 5 and 7 are consistent with the involvement of such cyclic binding and release of thiols in the fluctuations in the amounts of the five species measured here, though complete qualitative and quantitative agreement

between the changes in the various species is lacking. Specifically, transient increases in the content of mixed disulfides occur at the G_1/S transition and in G_2, at the times when minima occur in the contents of both QSH and PSSP (Fig. 7 B, C). (Quantitative comparison of the changes in these three species is not possible because of the uncertainties in the determinations, particularly of PSSP, as mentioned above, but it appears that the drop in PSSP content—which must be halved for comparison with the QSH and PSH contents—at 7.5 hr and during G_2, is smaller than the corresponding changes in the two remaining species.) In addition the PSH content rises at the time the PSSQ level increases at the end of G_1. More impressively, the fraction of the total disulfide content which appears as PSSQ increases markedly at the end of G_1, and a large increase occurs during G_2 as well (Fig. 7 A). Just such changes are expected on the basis of the model (27). While the present findings are thus in part interpretable in terms of cyclic fluctuations attendant on the conversion of intra- to intermolecular disulfide bonds, direct demonstration of polymerization is not afforded by the present study. Nevertheless, in view of the fact that the formation of arginine-rich histones (52) at the beginning of the S phase (6–8) and of mitotic spindle fibers during G_2 (27) involves both assembly of subunits (53) and increases in the number of intermolecular disulfide bridges (54–56), and in view of the fact that centriolar protein synthesis which commences at the beginning of S (3, 11) also involves subunit assembly (57), it seems reasonable to attribute the observed changes in PSH, PSSP, QSH, and PSSQ content partly to these processes.

Clearly, however, not all of the present findings are explainable in these terms. According to this model, the PSH content should show transient increases at the time and of the magnitude of the PSSQ increases, and should remain constant during the rest of the cycle. In fact the increase at the end of G_1 is larger than in PSSQ, it is followed by a prolonged and extensive drop (when normalized for protein content), and the fluctuations during G_2 do not correspond to those in PSSQ. Similarly, the model does not account for the large increase in QSH content that occurs during S (or for the drop at the end of G_2). Rather, the large changes in PSH and QSH levels which are apparent when the data are expressed in terms of a cell of constant protein content (or, approximately, constant mass or volume) mirror each other, and suggest that extensive conversion of the former to the latter takes place during the S phase. Such a change might be related to catabolism of sulfhydryl-rich proteins, possibly enzymes, that were synthesized during G_1 and G_2. It may be noted that the rate of synthesis of cellular proteins appears to increase during these latter phases of the cycle (Fig. 4 C), possibly reflecting rapid synthesis of enzymes. Whatever their cause, these large cyclical changes in the concentration of PSH and QSH implicate sulfhydryl groups in cellular metabolic processes other than—and additional to—the assembly of protein subunits. Identification of these processes will require further study.

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REFERENCES

1. HOWARD, A., and S. R. PELC. 1953. *Heredity.* 6(Suppl.):261.
2. PUCK, T. T., P. I. MARCUS, and S. J. CIECIURA. 1956. *J. Exp. Med.* 103:273.
3. STUBBLEFIELD, E., R. KLEVECZ, and L. DEAVEN. 1967. *J. Cell. Physiol.* 69:345.
4. PFEIFFER, S. E., and L. J. TOLMACH. 1968. *J. Cell. Physiol.* 71:77.
5. GAFFNEY, E. V., and R. N. NARDONE. 1968. *Exp. Cell Res.* 53:410.
6. LITTLEFIELD, J. W., and P. S. JACOBS. 1965. *Biochim. Biophys. Acta.* 108:652.
7. SPALDING, J., K. KAJIWARA, and G. C. MUELLER. 1966. *Proc. Nat. Acad. Sci.* 56:1535.
8. ROBBINS, E., and T. W. BORUN. 1967. *Proc. Nat. Acad. Sci.* 57:409.
9. DAVIES, L. M., J. H. PRIEST, and R. E. PRIEST. 1968. *Science (Washington).* 159:91.
10. BUELL, D. N., and J. L. FAHEY. 1969. *Science (Washington).* 164:1524.
10 *a.* KOLODNY, G. M., and P. R. GROSS. 1969. *Exp. Cell. Res.* 56:117.
11. ROBBINS, E., G. JENTZSCH, and A. MICALI. 1968. *J. Cell Biol.* 36:329.
12. BRENT, T. P., J. A. V. BUTLER, and A. R. CRATHORN. 1965. *Nature (London).* 207:176.
13. STUBBLEFIELD, E., and G. C. MUELLER. 1965. *Biochem. Biophys. Res. Commun.* 20:535.
14. STUBBLEFIELD, E., and S. MURPHREE. 1967. *Exp. Cell. Res.* 48:652.
15. LITTLEFIELD, J. W., A. P. MCGOVERN, and K. B. MARGESON. 1963. *Proc. Nat. Acad. Sci.* 49:102.
16. KLEVECZ, R. R., and F. H. RUDDLE. 1968. *Science (Washington).* 159:634.
17. VOLPE, P. 1969. *Biochem. Biophys. Res. Commun.* 34:190.
18. GELBARD, A. S., J. H. KIM, and A. G. PEREZ. 1969. *Biochim. Biophys. Acta.* 182:564.
18 *a.* MITCHISON, J. M. 1969. *Science (Washington).* 165:657.
19. GRIFFEN, M. J., and R. BER. 1969. *J. Cell. Biol.* 40: 297.
20. MADOC-JONES, H., and F. MAURO. 1968. *J. Cell. Physiol.* 72:185.
21. MAURO, F., H. MADOC-JONES, K. SAKOMOTO, and M. M. ELKIND. 1969. *Jap. J. Genet.* In press.
22. TERASIMA, T., and L. J. TOLMACH. 1961. *Nature (London).* 190:1210.
23. ELKIND, M. M., and G. F. WHITMORE. 1967. The Radiobiology of Cultured Mammalian Cells. Gordon and Breach, Science Publishers, Inc., New York. Chap. 3.
24. SINCLAIR, W. K. 1968. *Radiat. Res.* 33:620.
25. JUNG, C., and A. ROTHSTEIN. 1967. *J. Gen. Physiol.* 50:917.
26. RAPKINE, L. 1931. *Ann. Physiol. Physiochim. Biol.* 7:382.
27. MAZIA, D. 1961. *In* The Cell. J. Brachet and A. E. Mirsky, editors. Academic Press, Inc., New York. 3:77.
28. DAN, K. 1966. *In* Cell Synchrony. I. L. Cameron and G. M. Padilla, editors. Academic Press, Inc., New York. 307.
29. SANDRITTER, W., and A. KRYGIER. 1959. *Z. Krebsforsch.* 62:596.
30. TERASIMA, T., and L. J. TOLMACH. 1963. *Exp. Cell Res.* 30:344.
31. HAM, R. G., and T. T. PUCK. 1962. *Methods Enzymol.* 5:77.
32. MARCUS, P. I., and T. T. PUCK. 1958. *Virology.* 6:405.
33. OYAMA, V. I., and H. EAGLE. 1956. *Proc. Soc. Exp. Biol. Med.* 91:305.
34. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* 193:265.
35. MODIG, H. 1968. *Biochem. Pharmacol.* 17:177.
36. MOORE, S., R. D. COLE, G. GUNDLACH, and W. H. STEIN. 1960. *4th. Int. Cong. Biochem.* 8:52.
37. FASOLD, H., G. GUNDLACH, and S. DUPRÉ. 1961. *Biochem. Z.* 334:255.
38. CECIL, R., and J. R. MCPHEE. 1959. *Advan. Protein Chem.* 14:255.
39. KLOTZ, I. M., and B. R. CARVER. 1961. *Arch. Biochem. Biophys.* 95:540.
40. SALZMAN, N. P. 1959. *Biochim. Biophys. Acta.* 31:158.

41. TARNOWSKI, G. S., R. K. BARCLAY, I. M. MOUNTAIN, M. NAKAMURA, H. G. SATTERWHITE, and E. M. SOLNEY. 1965. *Arch. Biochem. Biophys.* **110**:210.
42. SCAIFE, J. F., and BROHÉE. 1969. *Can. J. Biochem.* **47**:237.
43. MODIG, H. G., and L. RÉVÉSZ. 1967. *Int. J. Radiat. Biol. Related Stud. Phys. Chem. Med.* **13**:469.
44. SCAIFE, J. J. 1964. *Can. J. Biochem.* **42**:1307.
45. HARRIS, J. W., and H. M. PATT. 1969. *Exp. Cell Res.* **56**:134.
46. HARRIS, J. W., R. B. PAINTER, and G. M. HAHN. 1969. *Int. J. Radiat. Biol. Related Stud. Phys. Chem. Med.* **15**:289.
47. PFEIFFER, S. E., and L. J. TOLMACH. 1967. *Nature (London).* **213**:139.
48. TOLMACH, L. J., T. TERASIMA, and R. A. PHILLIPS. 1965. *In* Cellular Radiation Biology. The Williams & Wilkins Co., Baltimore. 376.
49. MAURO, F., and M. M. ELKIND. 1968. *Cancer Res.* **28**:1150.
50. ELKIND, M. M., K. SAKAMOTO, and C. KAMPER. 1968. *Cell Tissue Kinet.* **1**:209.
51. BACQ, Z. M., and P. ALEXANDER. 1961. Fundamentals of Radiobiology. Pergamon Press, New York. Chapter 19.
52. ORD, M. G., and L. A. STOCKEN. 1963. *Nature (London).* **200**:136.
53. KIEFER, B., H. SAKAI, A. SOLARI, and D. MAZIA. 1966. *J. Mol. Biol.* **22**:964.
54. DEAKIN, H., M. G. ORD, and L. A. STOCKEN. 1963. *Biochem. J.* **89**:296.
55. SAKAI, H. 1966. *Biochim. Biophys. Acta.* **112**:235.
56. ELDJARN, L. 1965. *In* Cellular Radiation Biology. The Williams & Wilkins Co., Baltimore. 305.
57. LETTRÉ, H., and R. LETTRÉ. 1958. *Rev. Hématol.* **13**:337.