AN EVALUATION OF THE BARRNETT AND SELIGMAN PROCEDURE WHEN USED TO DETERMINE CHANGES IN THE RATIO OF NUCLEAR PROTEIN-BOUND DISULFIDE TO SULFHYDRYL GROUPS DURING MITOSIS[1]

B. B. HYDE[2]

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma

We have been using a modified Barrnett and Seligman SH procedure for relative quantitative determination of sulfhydryl proteins in the nuclei of plant meristems. Our aim is to determine whether the proportion of protein-bound S—S to SH in chromatin changes during the mitotic cycle. Reported here will be data accumulated in pursuit of this problem as well as data concerning the Barrnett and Seligman reaction as we use it on our material. We will show that this procedure should not be used on new material without considerable preliminary testing. After checking its reaction rate, effect of fixative, and several other factors, this procedure provides data which do suggest that the proportion of protein-bound S—S/SH in plant nuclei changes during the mitotic cycle.

MATERIALS AND METHODS

We have described our procedure in detail elsewhere (7): only an outline will be presented here. Onion root tips grown in aerated tap water are fixed in 3 parts ethanol to 1 part acetic acid. They are then embedded in paraffin and sectioned at 8 μ. In our earlier work sections were run through xylene, hydrated in an alcoholic series, and transferred directly from 30% ethanol to the DDD solution. Thus the opportunity for air oxidation of SH groups was minimized. In the series of experiments to be reported here, the sections were divided into two groups. Directly after the slides had been transferred from xylene to absolute alcohol, half were reduced two hours at 50°C. in a 0.5 M solution of thioglycolic acid in 0.4% KOH solution in absolute alcohol according to the method described by Teiger *et al.* (16). The other half remain in a control alcoholic solution containing 2 ml of 20% KOH per 100 ml of absolute alcohol. Both groups are then reacted with the sulfhydryl reagent, 2,2'-dihydroxy-6,6'-dinaphthyl disulfide (DDD) and azocoupled with Fast Blue RR. The intensity of the stain is measured by projecting the image on clear glass mounted in a 3¼ x 4¼ in. Leitz microscope camera. The area to be measured was centered on a very small circular opening in the window of the search unit. Thus it is possible to measure the density of an area smaller than the diameter of a metaphase chromosome. The size of the opening in the search unit was much smaller when metaphase chromosomes, nucleoli and telophase masses were measured than when interphase nuclei were measured. The interphase nucleus was larger in area but less homogenous than the other measured objects. The photometer is a Model 514M, battery powered, with a photomultiplier unit made by Photovolt Corp. The light source, a ribbon filament lamp controlled by a Sola constant voltage regulator, was filtered by a Photovolt interference filter having a peak transmission at 530 mμ. Measured nuclei were selected from the middle of median sections in the region of active cell division.

In our preparations of rapidly dividing plant tissues most of the SH stain before or after reduction is in the nucleus. Ernst and Hagen (5), by quite different methods (fractionation of cell components and amperometric titration) found that rat liver nuclei contain about 60% of the SH groups in the rat liver cell.

To test possible blocking of the DDD reaction by ribonucleic acid (RNA) present in the nucleus, sections fixed in 3:1 alcohol-acetic acid were treated with 1 mg/ml ribonuclease for two hours at 23°C, according to Swift (15). Control slides remained in distilled water. Then the DDD reaction was carried out as usual. No test for the effect of ribonuclease on thioglycolate reduction was made. The complete removal of RNA was checked using azure B.

OBSERVATIONS

The Proportion of S—S/SH at interphase, metaphase and telophase: Measurements taken from four representative experiments are tabulated in Table I. An earlier discussion of part of

[2] Address 1961–62 Division of Biology, California Institute of Technology, Pasadena, Cal.

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TABLE I

The proportion of protein bound S—S/SH in nucleolus and chromatin at three stages of mitosis as measured
by the Barrnett-Seligman histochemical procedure. Fixed in ethanol-acetic acid

	Exp. No.	Hours in DDD	Unreduced		Reduced in Thioglycolate		$\frac{b-a}{a}$
			Number of measurements	a Extinction	Number of measurements	b Extinction	
Nucleolus	208	1.5	35	.620 ± .008	38	.697 ± .010	.12
	229	1.25	42	.716 ± .012	20	.810 ± .007	.13
	233	3.00	10	.826 ± .021	10	.982 ± .021	.19
Metaphase	208	1.5	72	.460 ± .008	57	.624 ± .012	.35
	229	1.25	27	0.460 ± .006	23	0.538 ± .006	.17
	233	3.00	20	.667 ± .017	21	.786 ± .019	.15
Interphase	208	1.5	35	0.329 ± .008	38	0.476 ± .036	.45
	233	3.00	20	.583 ± .021	20	.735 ± .022	.26
	225	2.00	20	.557 ± .016	20	.742 ± 0.022	.33
Telophase	208	1.5	20	.886 ± .008	41	.879 ± .010	.00
	229	1.25	72	1.09 ± .006	40	1.07 ± .023	.00
	233	3.00	10	1.28 ± .073	10	1.31 ± .049	.00

this data has been published elsewhere (6). Most measurements of nucleolar sulfhydryl intensity are taken from the same nuclei as those from which interphase measurements were made. Thus the nucleolar data serve as a kind of control for the chromosome measurements; they provide some indication of the proportion of sulfhydryl groups in a protein other than that of chromatin. The nucleolus shows a reasonably consistent proportion of disulfide to sulfhydryl groups. Moreover, the data indicate a relatively small proportion of disulfide bonds in the fixed protein of the nucleolus. It is about one-half that reported by Teiger *et al.* (16) for the nucleolar protein of the starfish.

At interphase, when the living chromatin is thought to be metabolically active and in a highly hydrated state (11), consistent measurements are difficult to obtain. In the meristem of an onion root tip the nuclei vary in size and intensity of staining. For this reason it is difficult to interpret the rather large variation in proportion of S—S to SH groups in the sets of interphase measurements. Nevertheless, within any one experiment, the proportion of protein-bound S—S appears to be higher at interphase than in any stage of mitosis we have measured.

At telophase the chromatin stains intensely. The unreduced and reduced material do not differ significantly. Thus it can be inferred that virtually no S—S bonds exist in the chromatin at that stage of mitosis when the chromosomes are returning from the condensed to the extended state. Ap-

parently the structure of protein in chromatin is changing drastically at this stage because it can be seen that at metaphase as much as a third of the thiol sulfur appears to be in the oxidized state.

Using a slightly different modification of the Barrnett and Seligman procedure on HeLa cells, Sandritter and Krygier (12) obtained somewhat similar results. They found the nucleolus to have the highest extinction and the chromosomes to stain strongly during metaphase, anaphase and telophase. However, when studying the proportion of S—S/SH groups according to the Teiger *et al.* method, their results were meaningless, since post-reduction extinctions were lower than those obtained previous to reduction in nearly all parts of the cell.

Our findings, although suggestive, show that consistent and reliable results between experiments and with different materials can be difficult to achieve with this procedure. As we shall show below, it is probably not too strong to say that every SH- protein has its own characteristic reaction to this procedure. Further, relatively small variations in the technique will decisively affect the reaction rate.

Studies on reaction rate: In the four experiments recorded in Table I the tissues were incubated in DDD for different lengths of time. But no consistent relation was obtained between this time and the final intensity of the reaction. In an earlier paper (7) we suggested that the temperature of the reacting solution and length of incuba-

tion are important variables. With careful attention to the temperature of the reaction mixture results have become more consistent. We now incubate Coplin jars in a large thermostatically controlled water bath whose temperature remains at 50 ± 1°C.

Tests of the Barrnett and Seligman procedure showed that in our hands, a maximum stain was not obtained before 2.5–3.0 hours (see Fig. 10 in Hyde and Paliwal, 1959). However, the conditions of the technique are not quite the same when the thioglycolate reduction is carried out according to Teiger *et al.* Therefore, our newer work, reported here, is not entirely in accord with our earlier tests.

In particular, the control for the reduced material is held two hours at 50°C in absolute alcohol containing 2 ml of 20% KOH/100 ml. Reaction rate curves (Fig. 1 and 2) for this control show that five hours are required to complete the reaction under these conditions whether the material has been reduced in thioglycolate or not. The unreduced "control" curves thus take two hours longer to reach a maximum extinction than those in our earlier work when we did not require an alkaline-alcohol control step. These curves compared with those in our earlier work suggest strongly that disulfide lability is promoted by the alcoholic KOH solution. We did not, however, compare
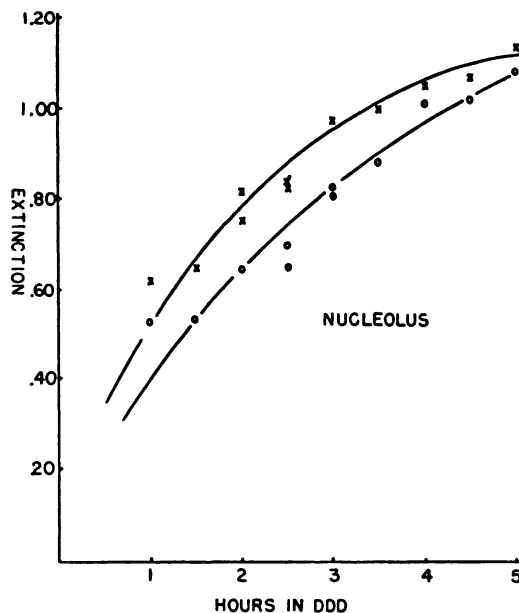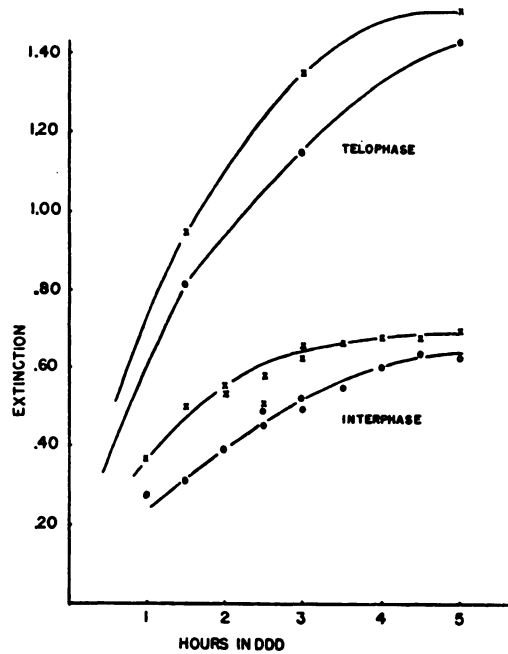

FIG. 2. Change in extinction of telophase and resting chromatin during the course of the DDD reaction. Crosses—material reduced in thioglycolate. Circles—control.

these extinctions with control material maintained at two hours in neutral alcohol.

It can easily be seen in Fig. 1 and 2 how minor changes in the conditions of the incubation might make drastic changes in the apparent proportion of S—S/SH in the nucleus or nucleolus. In Table II we have tabulated the ratios obtained by reading the curves (actual measurements are recorded in Table III) at three times, viz. 1.5, 3 and 5 hours, and shown how the proportion of S—S/SH is different at each time. Note that it is smallest at the 5 hour period. The proportion of S—S/SH stain (difference between reduced and unreduced extinctions divided by the unreduced extinction) gradually decreases from 1–3 hours and then decreases more rapidly as the curves level off.

Several additional points about these curves need to be made here. By the five hour period when the reaction has run its course (Table III), none of the nuclear components show statistically significant differences between reduced and unreduced extinctions. It will also be noticed that the unreduced curve gradually approaches the reduced curve. It therefore seems probable, as we suggested in our earlier report, that the alkaline DDD reaction mixture actually makes S—S bonds reactive to DDD. Moreover, the alkaline-alcohol "control" for the thioglycolate reduction appears

FIG. 1. Change in extinction of nucleoli during course of DDD reaction. Crosses-material reduced in thioglycolate. Circles—control material.

also to facilitate the opening of S—S bonds since the absolute amount of stain in the sections measured for the present report is greater than that in our earlier work where no alkaline-alcohol step was used.

It thus seems fair to infer that the shape of these curves is actually determined by the sum of two reactions. The first is a straightforward reaction of DDD with available SH groups. The second, which is affected by alkalinity, is the opening of S—S bonds. This latter reaction, although probably slower, introduces serious problems to the interpretation of data in which TGA-reduced and unreduced material is to be compared. It seems doubtful that the second reaction can be eliminated by changing the nature of the reducing mixture and its control, or by modifying the solution containing DDD. Our solution to this problem has been to stop the reaction at three hours when the reduced and unreduced curves are appre-

ciably different. A two hour incubation time would clearly produce an even greater difference but since at this time the curves are rising most rapidly, minor fluctuations in technique would have the greatest effect on variations between experiments. Another possible solution to this problem is considered in the next section.

Effect of fixative on the reduction reaction: The effect of a variety of fixatives on the density of the SH stain has been reported elsewhere (7). It was shown that the fixative affected the intensity of stain as well as the rate of reaction. That work led to the adoption of 3:1 alcohol-acetic acid as a standard in our studies.

Several more fixatives have been tested recently, but this time for their effect on the reduction reaction. All of these mixtures and their components are discussed by Baker (1) and the proportions used are suggested in his treatise. Since 10% trichloroacetic acid (TCA) and 3:1 alcohol: acetic acid have been tested in our previous work, they have been used again for comparison. The results may be seen in Table III.

The fixative affects the outcome of the reduction process remarkably. For example, thioglycolate reduction after TCA fixation induces relatively little increase in staining intensity in any component of the cell measured. It is pertinent to point out here that Sandritter and Krygier obtained their paradoxical results on material fixed in 1% TCA in 80% ethanol.

On the other hand, 5% acetic acid (HAc) appears to fix the nucleolus quite differently from the resting chromatin when their respective sulfur-containing proteins are observed by this method. The contrast between them is greater

TABLE II

Change in proportion of protein-bound S—S/SH during the course of the DDD reaction. The proportions are determined from the curves in Fig. 1 and 2 and are not taken from actual readings except in the case of telophase curve

	Time in Hours		
	1.5	3.0	5.0
Nucleolus	.26	.19	.04
Resting chromatin	.50	.22	.095
Telophase	.15	.17	.06

TABLE III

Effect of fixative on change in extinction of Barrnett and Seligman reaction following Thioglycolate reduction. Except where indicated, time of incubation in DDD was 3 hours

Fixative	Nucleolus			Resting Nucleus			Telophase Chromatin		
	Control	Thioglycolate Reduced	S—S stain SH stain	Control	Thioglycolate Reduced	S—S stain SH stain	Control	Thioglycolate Reduced	S—S stain SH stain
3 Ethanol:1 acetic acid, 1.5 hrs	.539 ± .017	.643 ± .017	.19	.308 ± .015	.503 ± .012	.33	.819 ± .026	.944 ± .023	.15
3 Ethanol:1 acetic acid, 3.0 hrs	.827 ± .031	1.15 ± .024	.39	.499 ± .026	.659 ± .011	.22	1.15 ± .028	1.37 ± .039	.11
3 Ethanol:1 acetic acid, 5.0 hrs	1.08 ± .027	1.13 ± .019	.046	.624 ± .023	.695 ± .027	.11	1.41 ± .036	1.49 ± .029	.065
10% TCA	.834 ± .024	.901 ± .039	.08	.522 ± .024	.581 ± .074	.11	1.02 ± .097	1.17 ± .014	.15
5% Acetic acid	.540 ± .026	.562 ± .036	.04	.384 ± .039	.540 ± .030	.35	1.126 ± .098	1.426 ± .017	.27
Sat'd HgCl₂	1.35 ± .061	1.20 ± .035	Decrease	.966 ± .031	.853 ± .022	Decrease			
95% Sat'd HgCl₂, 5% acetic acid	1.17 ± .046	1.09 ± .010	Decrease	.694 ± .017	.673 ± .036	Decrease			
3 Ethanol:1 acetic acid, sat'd HgCl₂	1.03 ± .020	1.28 ± .049	.24	.572 ± .017	.869 ± .022	.52	1.32 ± .079	1.38 ± .049	.045

than with any other fixative. Acetic acid is said (1) to be a good nucleic acid precipitant but not a good protein precipitant. Since, in onion root tips, the nucleolus contains a relatively large amount of RNA as compared to the resting chromatin, the difference may be related to the presence of this nucleic acid. It will be noticed that extinction values are low in the case of the nucleolus and nucleus although in telophase they are surprisingly high. An explanation discussed in the next section is the possibility that RNA blocks the DDD reaction. If acetic acid is a good nucleic acid precipitant its action may be to inhibit the reactivity of the RNA-associated protein.

Another explanation for the effect of acetic acid fixation may lie in its reaction with deoxyribonucleoprotein. The remarkable increase in stain (35% and 27% respectively), produced by reducing resting nuclei and telophase chromatin fixed in 5% HAc may be in part due to the splitting of deoxyribonucleic acid (DNA) from its protein by the acetate ion (1). While histone, the protein normally associated with DNA, has essentially no cysteine (4), other proteins have been uncovered when DNA is removed by enzymatic methods (8). It is clear that a higher proportion of disulfides in chromatin is susceptible to reduction by thioglycolate after acetic acid fixation than after any other fixative we have tried.

Another fixative found to produce unexpected results in our earlier work is saturated mercuric chloride. Table III shows that following HgCl₂ fixation: (a) extinction values in all parts of the cell are high and (b) reduction produces no significant change in extinctions and indeed seems consistently to reduce the intensity of stain. We infer, then, that saturated mercuric chloride coagulates nucleoproteins in such a way that disulfides can readily react with DDD without reduction, or that it attacks disulfide links. Baker says that HgCl₂ is not a powerful coagulant of nucleoprotein and notes that it unmasks lipoprotein. In our experiments it surely has the effect of unmasking and making reactive both S—S and SH.

We have tried HgCl₂ in two combinations: with 5% acetic acid and with 3:1 alcohol-acetic acid. In the former case the results are not unlike HgCl₂ alone except that, in resting chromatin particularly, the reaction may not be quite so strong. The HgCl₂ may actually block SH groups in this more acid combination, and, moreover, the nucleic acids which should be more effectively precipitated by this combination may also be inhibiting the stain to some extent.

In the last fluid, HgCl₂ was dissolved in a mixture of 3 parts 95% ethanol and 1 part glacial acetic acid. It is much more soluble in this mixture

than in water and has a definite influence on the outcome of the DDD reaction. The nucleolus and resting nucleus (compare to 3 hour alcohol-acetic acid fixation) respond with increased extinctions in both the unreduced and reduced material as well as by a greater proportional change resulting from reduction. The telophase measurements are similar to those obtained after alcohol-acetic fixation.

It is possible that this mixture might be a good one for the DDD procedure. Its intensification of the SH reaction might be accomplished in three ways. First, it might make insoluble a greater proportion of the original nuclear protein. Secondly, its action may make the tissue protein more resistent to hydrolysis during the long periods of incubation in alkaline-alcoholic reaction mixtures. Thirdly, it may also preserve protein in such a way that more of the SH groups are unmasked and therefore reactive. The increased effect of reduction following fixation in this mixture may be due to the same cause. That is, the protein of the nucleolus and resting chromatin is preserved by this mixture in such a way that more S—S bonds are unmasked and therefore are reduced by thioglycolate. It should not, however, be used without making further reaction rate tests such as we have made for alcohol-acetic acid alone. A priori, one cannot help suspecting the use of mercury-containing fixatives in a histochemical procedure for SH groups.

The effect of ribonuclease: This experiment was made to test the possibility that RNA might mask or make reactive certain SH groups in the protein of the nucleus. The test did not concern S—S bonds. The results may be seen in Table IV.

The "t test" indicates that only in the nucleolus does a significant increase in extinction result from removal of RNA. While some doubt exists as to the proportions of RNA to protein in the nucleolus, it is certainly more concentrated there than in any other part of the nucleus. Further, the RNA may be more tightly bound to protein in the

TABLE IV

Effect of ribonuclease on the reaction of nucleoprotein to cytochemical test for protein-bound SH

	Extinction	
	Control	RNAase
Nucleolus	.749 ± .020	.888 ± .020
Metaphase	.419 ± .017	.464 ± .014
Interphase	.380 ± .014	.439 ± .021
Telophase	1.05	1.05

nucleolus than elsewhere in the nucleus. Bell (2) has suggested that SH groups may be involved in the linkage of RNA to its protein, but only in the cytoplasm.

In the case of metaphase chromatin and the resting nucleus, increases resulted from ribonuclease treatment, but they are not statistically significant. It is with the sulfur-containing or residual protein of chromosomes that RNA is normally considered to be associated, not the histone, which has little or no cystine. Nonetheless, the data make it reasonable to suspect that RNA does suppress the reactivity of an appreciable number of nuclear SH groups other than those in the nucleolus. Ribonuclease (RNAase) has no effect on telophase chromatin, which is shown by our other data to have the lowest proportion of S—S bonds.

DISCUSSION

While the Barrnett and Seligman procedure may have some limitations when used to determine SH and S—S quantitatively, it does appear to provide consistent answers to certain questions. It shows that the sulfur-containing protein of chromatin at three different stages of mitosis are different from each other in their response to thioglycolate reduction. With several fixatives and reaction conditions telophase chromatin yields the lowest ratio of S—S bonds, the resting nucleus the highest ratio of S—S bonds, and the metaphase chromosomes are intermediate. Two fixatives (10% TCA and 5% acetic acid) result in a higher ratio of S—S at telophase and have already been discussed. These histochemically determined differences may not, indeed probably do not, result from measurement of all SH and S—S groups. Nor does the proportion reflect the real one necessarily, for we have presented evidence here and previously (7) that the DDD reagent and some fixatives may cleave some S—S bonds which then behave as SH groups.

Nevertheless, the fact that chromosomal protein in various stages of mitosis reacts differently to the same fixation, SH reaction, and thioglycolate reduction procedure, indicates changes in the sulfur proteins. The possible changes are many, since this protein fraction is the most variable in the nucleus and the least well characterized. For example, it is probable that SH protein not only makes up part of the basic structure of the chromosome—the residual chromosome—but also, at least in the metabolic nucleus, may be produced as part of its genetic

activity. Schurin (13) has shown for example, that RNA and non-histone protein are synthesized by certain "puffs" in the salivary gland chromosomes of *Drosophila virilis*. If complete ribosomes are assembled in the nucleus, the protein which they contain is included in our measurements. Richards (10) has shown that a large portion (70% in one plant species) of the dry mass of the prophase nucleus is not included in the chromosomes at metaphase. Thus the protein in metaphase chromosomes may be different from that of prophase both in structure and kind.

At telophase some of the protein of the spindle is included. At this stage the spindle is also losing its organization presumably by the breaking down of S—S bonds (9). Thus the spindle protein would be expected to have a low S—S/SH ratio at this time. However, the spindle does not stain strongly compared to the chromatin at this stage and certainly does not compose a major portion of the protein we are measuring.

The general trend indicated by our measurements, viz., the highest proportion of S—S/SH at resting stage, a moderate decrease toward metaphase, followed by a sharp decrease to an extremely low ratio at telophase, parallels the change in protein-bound SH for the cell as a whole. Stern (16) reports that in isolated lily microspores protein S—S groups gradually decrease during prophase, reach a low point at the end of mitosis, and then rise sharply.

SUMMARY

Using a modification of the Barrnett and Seligman histochemical procedure, the proportion of protein-bound S—S/SH was measured in the chromatin of onion root tip cells at three different stages of mitosis as well as in the nucleolus. Inconsistencies between experiments led to studies of the nature of the histochemical reaction before and after sections were reduced in thioglycolate. Different fixatives and ribonuclease were tested for their effects on the outcome of the reaction.

Some deviation from the change in S—S/SH ratio during mitosis found after ethanol-acetic fixation occurs in material fixed in 10% trichloroacetic acid, 5% acetic acid and saturated $HgCl_2$. Ribonuclease treatment was found to increase significantly SH stain in the nucleolus, but not significantly in the resting nucleus, metaphase,

or telophase chromatin. The possibility that nucleic acids may modify the SH reaction after 5% acetic acid fixation is raised. Further, the data are interpreted to indicate that saturated $HgCl_2$ as well as the histochemical reaction itself makes disulfide bonds stainable in the absence of reduction.

After fixation in standard ethanol-acetic acid mixtures the S—S/SH ratio is highest at interphase, lowest at telophase, and intermediate at metaphase.

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