due to a late stimulation effect after the medium change. From cultures synchronized by the double thymidine method, cells were prepared for measuring with the flow microfluorometer at short intervals after release from the last TdR-block. As shown in Figure 2, A–F, cells are in G_1 at 1 h with no detectable G_2+M-phase cells. Over the next few hours it can be seen that whole G_1-cell population synchronously entered the S-phase and subsequently the G_2-phase. The G_1-peak disappeared first but reappeared 8 h after release from the TdR-block, when the cells had gone through mitosis, as was established by counting cell numbers. The Table gives the distribution through the cell cycle phases expressed as percentages.

Discussion. Changes in the DNA-distribution pattern registered by cytophotometric methods were observed in animal cells after treatment with cytostatica[6,8]. Preliminary experiments with HeLa-monolayer cells have indicated that cell synchrony can easily be demonstrated using the methods presented in this report for HeLa-cell suspension.

The synchrony which follows removal of an excess thymidine block is observed as a complete change in the pattern of DNA-distribution within a few hours (Figure 2). Although the cells start from the same point in G_1, a small fraction reaches the next G_1-phase while some cells are still in the first G_2-phase, i.e. there is a gradual decrease in the degree of sychrony, a common observation in synchronized tissue cultures.

No such changes in the pattern of DNA-distribution were observed in asynchronous populations (Figure 1).

The flow of cells through the cell cycle can therefore easily be monitored by changes in ethidium bromide staining of cells. For correct interpretation of these results, one must assume that ethidium bromide binds stoichiometrically to the DNA. The percentage of cells in the S-phase was used to obtain a measure of the degree of synchrony. In earlier studies[9], however, the percentage of S-phase cells in asynchronous populations as monitored by autoradiography was about as high as that obtained from cytophotometric measurements (extrapolation of accumulated DNA-frequencies). These differences can be attributed to the deficiencies in the mathematic method applied. Because of the very small differences in DNA-content of early or late S-phase cells from those in G_1 or G_2+M, difficulties arise in the differentiation of the particular cell cycle phase. No other method (see also[6]) for the processing of results exists at the present time.

Zusammenfassung. Die schnelle fluoreszenzphotometrische Methode wurde zur Bestimmung der Synchronisation des Zellzyklus Thymidin-synchronisierter HeLa-Zellen verwendet. Das Verteilungsmuster der verschiedenen Zellzyklusphasen konnte näherungsweise bestimmt werden.

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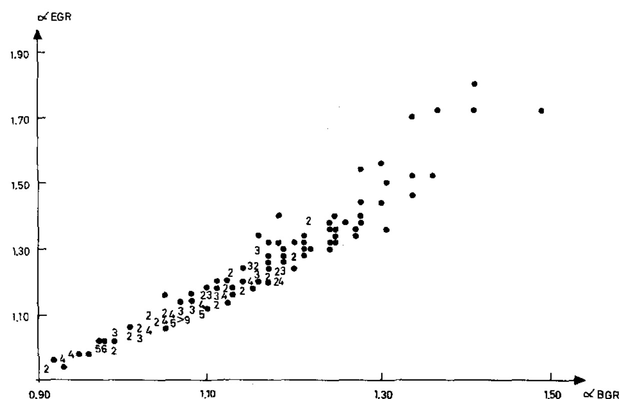
Glutathione Reductase Test with Whole Blood, a Convenient Procedure for the Assessment of the Riboflavin Status in Humans

Erythrocytic $NADPH_2$-dependent glutathione reductase (EGR) has been employed as a parameter for the evaluation of the riboflavin status in humans[1]. For field studies and investigations with children, there is, however, some request to cut down the amount of blood used for the original EGR test, and THURNHAM et al.[2] developed a micromethod separating erythrocytes in heparinized capillary tubes.



Stimulation of EGR and BGR for the blood drawn from 203 pregnant women.

Another approach, which has been tried for transketolase by DREYFUS[3], could be the use of whole blood, without separating erythrocytes. The main part of glutathione reductase activity of the blood is in the red cells and, if there is no interference with the assay by substances in the plasma, the procedure seems reasonable.

Recently, the suitability of whole blood for the glutathione reductase test has been shown with rats on a varying riboflavin intake[4]. This communication shows the application of the blood glutathione reductase (BGR) test for humans, and its relation to the EGR test and flavin levels in whole blood.

Materials and methods. Whole blood[5] (stabilized with ACD[6] or heparin) was applied in the BGR test, which differs from the original EGR test[6] mainly in using only 0.05 ml of native or stabilized blood in 9 ml 0.1 *M* phosphate buffer, pH 7.4, enriched with 0.1% $EDTA\text{-}Na_2$; this suspension can be stored frozen for up to several

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Table I. Blood glutathione reductase assay

	Cuvette 1		Cuvette 2	
	ml	mole/cuvette	ml	mole/cuvette
Blood hemolysate 1:185	1.5		1.5	
Containing phosphate buffer, pH 7.4,		1.5×10^{-4}		1.5×10^{-4}
EDTA-Na_2		4×10^{-6}		4×10^{-6}
$NADPH_2$	0.1	2×10^{-7} [a]	0.1	2×10^{-7} [a]
FAD	0	—	0.05	1.5×10^{-8}
H_2O	0.05	—	0	—
	Preincubation at 37 °C for 5 min in both cuvettes			
GSSG	0.05	3.8×10^{-7}	0.05	3.8×10^{-7}
	Incubation at 37 °C, measuring of decrease of absorbance at 334 nm for 9 min (ΔA_1 for cuvette 1, ΔA_2 for cuvette 2)			

Enzyme reaction: GSSG+$NADPH_2 \rightleftharpoons$ 2GSH+NADP. Activation coefficient: $\alpha_{BGR} = \Delta A_2/\Delta A_1$. Calculation of enzyme activities on the basis of $\varepsilon_{334\,nm} = 6 \times 10^6$ cm²/mole for $NADPH_2 \rightarrow NADP$, hemolysate 1:185, hemolysate volume h = 1.5 ml, total volume in the cuvette v = 1.7 ml, reaction time t = 9 min. E.g.:

$$a \text{ (per g Hb)} = \frac{1.7}{1.5} \cdot \frac{185}{6 \cdot 9} \cdot \frac{\Delta A \cdot 100}{\text{Hb [g\%]}}$$

$$a_o \text{ (per g Hb)} = 388 \cdot \frac{\Delta A_1}{\text{Hb [g\%]}}; \quad a_+ \text{(per g Hb)} = 388 \cdot \frac{\Delta A_2}{\text{Hb [g\%]}}$$

[a] Calculated for 100% $NADPH_2$. The following abbreviations are used: NADP, oxidized form of nicotinamide adenine dinucleotide phosphate; $NADPH_2$, reduced form of nicotinamide adenine dinucleotide phosphate; BGR, $NADPH_2$-dependent glutathione reductase (E.C. 1.6.4.2) in whole blood; EDTA-Na_2, disodium ethylenediamine-tetra-acetate; FAD, flavin adenine dinucleotide; GSSG, oxidized form of glutathione; GSH, reduced form of glutathione; Hb, hemoglobin.

months below −15 °C until a convenient time for analysis. Hemolysis was accomplished by addition of 0.2 ml saponin solution (1%) after thawing. Cell débris was separated by centrifugation. The assay was performed at 37 °C according to the procedure given in Table I.

The EGR test using red cell hemolysates was applied to the blood samples from schoolchildren, as described earlier[6]. In the case of pregnant women, venous blood was drawn in a heparinized vacutainer. Plasma and red blood cells were separated and the erythrocytes were stored in ACD stabilizer[6] up to 3 days in the refrigerator until hemolysis and subsequent analysis.

Flavin analysis in whole blood (0.3 ml) was in principle carried out according to the method of BURCH et al.[7].

Results and discussion. For the evaluation of the EGR test, the stimulation of the enzyme activity by added coenzyme had been chosen by us[6] rather than the enzyme activities as such, in order to eliminate or diminish the influence of those effects on enzyme activity which are unrelated to the riboflavin status. This stimulation

Table II. Glutathione reductase activities a_o (without FAD addition) and a_+ (with FAD addition) of whole blood (BGR), activation coefficients α for whole blood (α_{BGR}) and erythrocytes (α_{EGR}) and blood flavin levels, f_B (nmole/100 ml blood), for pregnant women

	Minimum	Maximum	n	Mean	Standard deviation
a_o (BGR, per g Hb)	4.28	9.04	195	6.23	0.995
a_o (BGR, per ml erythrocytes)	1.35	2.94	195	2.00	0.327
a_o (BGR, per l blood)	457.8	1171.8	203	750.4	132.9
a_o (BGR, per 10^{11} erythrocytes)	12.11	28.14	195	18.65	3.26
a_+ (BGR, per g Hb)	5.13	10.03	195	6.83	0.815
a_+ (BGR, per ml erythrocytes)	1.65	3.10	195	2.20	0.255
a_+ (BGR, per l blood)	630.5	1175.6	203	821.4	97.5
a_+ (BGR, per 10^{11} erythrocytes)	15.15	28.81	195	20.39	2.29
α_{BGR}	0.91	1.49	203	1.11	0.107
α_{EGR}	0.94	1.79	203	1.18	0.156
f_B	19.7	51.9	199	32.6	5.84

Values for a_o and a_+ are given as µmole substrate converted per min per g hemoglobin of native blood, per ml erythrocytes (calculated from the hematocrit value of native blood), per l blood and per 10^{11} erythrocytes.

expressed as the activation coefficient $\alpha = a_+/a_o$ has proved useful as an index of the riboflavin status in the case of EGR and has been tried also for whole blood in animals[4].

For the blood samples from 45 schoolchildren the activation coefficients for the stimulation of the glutathione reductase in erythrocytes ($0.96 \leq \alpha_{EGR} \leq 1.46$) and whole blood ($0.92 \leq \alpha_{BGR} \leq 1.28$) showed a good correlation with a coefficient $r = 0.96$ (Pearson-Bravais).

The Figure presents the stimulation of EGR and BGR for the blood drawn from 203 pregnant women. For the two collectives, very similar equations between the activation coefficients were calculated:

$$\text{Schoolchildren} \quad \alpha_{BGR} = 0.67\,\alpha_{EGR} + 0.29$$

$$\text{Pregnant women} \quad \alpha_{BGR} = 0.67\,\alpha_{EGR} + 0.33$$

Because of the increased stability in our test system of glutathione reductase from whole blood as compared to erythrocytes, it appears reasonable to calculate activities for the BGR. One may argue about the proper basis for the activities to rely on, especially when there are anaemic persons in the group investigated, as was the case with the pregnant women (DECKER, GLATZLE, HINSELMANN, to be published).

Whether the activities were based on hemoglobin[4], hematocrit, blood volume or erythrocytes, the activities a_o (without FAD addition) were negatively correlated with α_{BGR} (Tables II and III). This means that, in the case of high activation coefficients, the enzyme activities a_o of the flavoenzyme glutathione reductase were low, indicating a biochemical riboflavin deficiency. On the hemoglobin basis, considering the correlation coefficients r (Pearson-Bravais) in Table III, there was no statistically significant correlation between the activities a_+ (after FAD addition) and the activation coefficients α_{BGR}. On the other hand, a negative correlation was found between α and a_+ when the activities were calculated for 1 l of blood, 1 ml of erythrocytes or 10^{11} erythrocytes, respectively. Depending on the basis for the activities, this might point towards either no or some loss of glutathione reductase protein (apoenzyme plus holoenzyme) in riboflavin deficiency[4]. More work has to be done to elucidate this problem which is complicated by various circumstances affecting glutathione reductase activity[8].

In cross-sectional studies – contrary to controlled ones – SHARADA and BAMJI[9] reported that with their test system a correlation between glutathione reductase activity and red cell flavin levels is often not seen. In our study with pregnant women, we found statistically significant, albeit not high, correlations between flavin levels in whole blood and the enzyme parameters α_{EGR}, α_{BGR} and a_o which are presented in Table III and were also established by the Kendall rank correlation test: Low enzyme activities a_o – or high activation coefficients – corresponded to low flavin levels in blood and high enzyme activities a_o – or low activation coefficients – to high flavin levels.

Table III. Correlation coefficients r (Pearson-Bravais) for blood and erythrocyte glutathione reductase and blood flavin ($191 \leqq n \leqq 203$)

	α_{BGR}	f_B
a_o (BGR, per g Hb)	−0.67	+0.33
a_o (BGR, per ml erythrocytes)	−0.71	+0.34
a_o (BGR, per l blood)	−0.78	+0.52
a_o (BGR, per 10^{11} erythrocytes)	−0.79	+0.36
a_+ (BGR per g Hb)	−0.11 n.s.	+0.13 n.s.
a_+ (BGR per ml erythrocytes)	−0.19	+0.16
a_+ (BGR per l blood)	−0.39	+0.47
a_+ (BGR per 10^{11} erythrocytes)	−0.41	+0.23
α_{BGR}		−0.39
α_{EGR}	+0.96	−0.36

—, Significant; n.s., not significant.

Zusammenfassung. Eine vereinfachte Variante des Glutathionreduktasetests zur Erfassung des Riboflavinstatus wird beschrieben, für die nur 0,05 ml Gesamtblut anstelle von gewaschenen Erythrozyten benötigt werden. Beim Vergleich der Stimulierbarkeit der Glutathionreduktase durch das Coenzym FAD ergab sich eine sehr gute Korrelation zwischen den Aktivierungskoeffizienten bei Verwendung von Gesamtblut und Erythrozyten. Diese zeigten statistisch signifikante Korrelationen zum Flavingehalt im Blut.

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Aqueous Solution of Acridine Orange in the Staining of DNA-Aldehyde

The attempt to stain DNA-aldehyde in tissue sections with the fluorescent dye, acridine orange (AO) following Feulgen procedure led to unsuccessful results. The present communication embodies results of a study with acridine orange and its use in the staining of acid hydrolyzed DNA.

Two samples of AO were used in this investigation. One was manufactured by the National Aniline Division, New York, USA, batch No. 11538 and the other by G. T. GURR, London, batch No. 55744. It is a basic dye (C.I. No. 46005) of the acridine group with the following structural formula: