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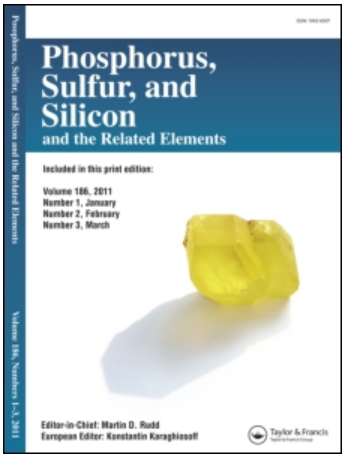

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INTRACELLULAR SH-LEVELS AS RELATED TO TUMOR PROBLEMS

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INTRACELLULAR SH-LEVELS AS RELATED TO TUMOR PROBLEMS

by

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CONTENTS

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I. GENERAL DEFINITIONS OF INTRACELLULAR THIOLS AND SHORT DESCRIPTIONS OF THEIR BIOCHEMICAL AND BIOLOGICAL FUNCTIONS

In the following review we will distinguish between non-protein thiols NPSH and protein thiols PSH. NPSH include low molecular SH-compounds such as glutathione, cysteine, SH containing coenzymes (such as coenzyme A, dihydrolipoic acid and glutathione). It cannot be the goal of this review to deal with the biochemical functions of CySH or the coenzymes which belong to elementary biochemistry.

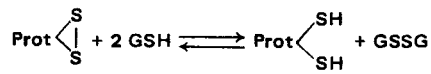
Glutathione (GSH) represents, in most cell types, the main constituent of NPSH as for example, Schindler[1] could show in rat kidney liver jejunum and Ehrlich ascites tumor cells (EATC).

TABLE I

NPSH and GSH contents of rat organs and of EATC, determined with DTNB and *o*-phthalaldehyde, according to Schindler[1]

Rat Organ	GSH	NPSH (μM/g w.w.)
Kidney	1.31± 0.21	1.11 ± 0.15
Liver	4.68 ± 0.62	5.07 ± 0.22
Jejunum	2.14 ± 0.33	2.09 ± 0.36 Calcutt *et al.*[2]
EATC	2.69 ± 0.46	2.27 ± 0.43

One of the most important properties of GSH lies in its participation in redox equilibrium with GSSG wherefrom probably can be derived its role as an important activator for SH-functional enzymes and for mitosis. The latter effect has been ascribed by Mazia[3] to a reduction of S–S-bridges of proteins of the mitotic apparatus to SH-groups which stimulate mitotic activity:



GSH also acts as an acceptor for α,β-unsaturated carbonyl compounds and is added by means of specific alkene-transferases of the liver to the α,β-double bond of such substances, which is the first step in detoxification and renal excretion mechanism.[4]

As it will be shown later, NPSH–primarily GSH–can act as a protector of PSH against the attack of SH-reactive substances and also against radiation injury.[5]

Let us mention that the hydrogen of GSH can be enzymatically transferred to different substrates and

that GSH plays an important role as a cofactor for enzymes (isomerases, glyoxalase) and as a prosthetic group, *e.g.*, in triosephosphate dehydrogenase.[6] For further information excellent surveys, citations 3, 5, 6, and 7, are recommended.

Protein-bound SH-groups (PSH)

The majority of intracellular PSH is associated with soluble proteins (symbol: PSH_L), the minor part with insoluble structural proteins (symbol: PSH_S). PSH_L belong to proteins of rather general importance and functions such as albumins and globulins of the α- and β-types as well as to proteins of highly specific functions, particularly enzymes (most of the dehydrogenases, phosphokinases, decarboxylases, protein-synthetase-, DNA-synthetase- and fatty acid synthetase-system etc.).

Special correlations to the problems of malignant tumors exist in the following views:

In malignant tumors–as generally in actively dividing cells–the activity of the DNA-synthetase system is increased.[8] This system having itself SH-groups of functional importance[8a] probably may be stimulated by GSH synthetized specially favoured by an active glycolysis (*cf.*, p. 203).

The stimulating effect of GSH to proteins of the mitotic apparatus has already been mentioned, as well as the possibility of transfer of H from SH to S–S-groups of mitotic proteins. It should be mentioned here that also SH-groups of low molecular TCA-soluble nuclear proteins can enter SH $\leftrightarrows$ S–S–exchange equilibria and thus may be a cause for the well-known observation that before mitosis decrease of low molecular thiols occurs synchronously with increase of high molecular thiols and corresponding decrease of high molecular disulfide groups.[9]

In a more recent review, Sakai[9a] points to the existence of SH $\leftrightarrows$ S–S-exchange equilibria also between macromolecular cellular proteins, especially a contractile protein (CP) of the cortex of sea urchin egg cells and a protein of the mitotic apparatus (CIP) which can be precipitated by Ca^{++}. A maximum of SH-content of CP accompanied by a minimum of SH-level of CIP is the prerequisite for the onset of cell kinesis. Such a mechanism has not been established for all known animal cells but seems to be likely.

Some detailed observations may be mentioned here in order to indicate the kind of nuclear proteins involved and the different stages of cell life at which they act (*cf.* Fig. I):

Nuclear thiol groups were found in one of the five histone fractions, but are mainly located in non-histone proteins, the so-called acidic residual proteins.[9b]

According to Hilton[10] there exist SH-containing fractions of nucleohistones having a derepressing effect on nucleic acid synthesis before mitosis starts. Wang[11] reported that DNA-directed RNA-synthesis occurs at binding sites between DNA and nuclear SH-proteins where that DNA is in an active state. Sandritter[12] found that in synchronized cells of human cancer (HeLa) protein-SH levels increase before pro-phase and decrease in telo-phase whereas GSH remains constant. Mazia[13] observed the appearance of protein-SH in the spindles in pro-phase and in spindles, asters and chromosomes in meta-phase. During mitosis SH-containing histones rise, as Ord[14] has found in sea-urchin eggs.

Summarizing, we can say that in the nucleus of many cell types, including mammals,[15] there exist SH-rich macromolecular proteins. Till now we still have poor information about the chemical nature of the cellular thiols involved in cell growth, division and differentiation. For example, the nuclear thiols appearing periodically in division of synchronous sea urchin eggs[13] have never been identified, as Gronow *et al.*[9b] stated recently These authors make successful progress in elucidating this fascinating problem. One group of proteins is soluble in water or low ionic strength base, another soluble in KCl.[16] These proteins are at least partly associated with arginine-rich histones. According to Ord and Stocken,[17] the thiol groups of nucleohistones are important both for the structural alterations of chromatin during mitosis and for initiation of nucleic acid synthesis. They are also bound to DNA being not only of indispensable importance for structural integrity of DNA and chromosomes[18] but also being a stimulator for DNA duplication and DNA-directed RNA-synthesis and thus for cell division.

Thiols of nucleoproteins increase during normal growth and especially in tumors.[19] Lea *et al.*[15a] recently found that acidic nucleoproteins increase in correlation with the growth rate of rat hepatoma Gronow *et al.*[15b] communicate that SH-containing acidic nucleoproteins increase during development of diethylnitrosamine induced rat hepatoma. According to Stein *et al.*[15c], chromatine from rapidly growing hepatoma has increased contents of non-histone proteins. Thus it is understandable that these SH-containing "residual" nucleoproteins are the target of the attacks of tumor therapeutics, such as antimetabolites, SH-inhibitors and also of X-rays[19] and that correlations have been found between the ratio protein-SH/soluble SH of animal tumors and their sensitivity to an alkylating reagent.[20, 20a]

SH-containing non-histone proteins attached to the chromosomal DNA stimulate the cell division. Therefore, division can be inhibited by blocking of these

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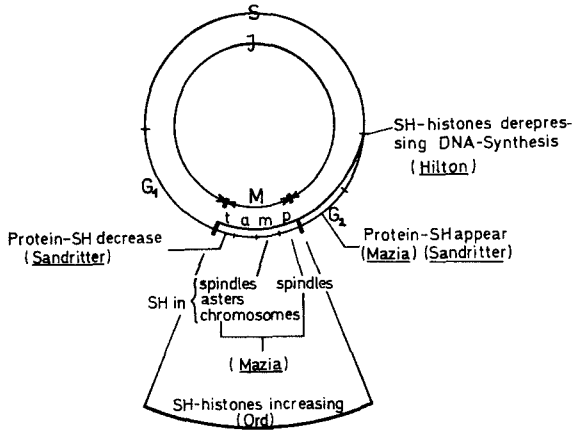

FIGURE IA

The cycle of the cell life. I = Inter- or resting phase ($G_1 + S + G_2$); S = synthesis phase at the end of which DNA-reduplication starts. M = Mitosis [p (pro-) + m (meta-) + t (telo-) + a (ana-)-phase]. Duplicated line from the end of S to G_1 symbolizes reduplication of DNA.

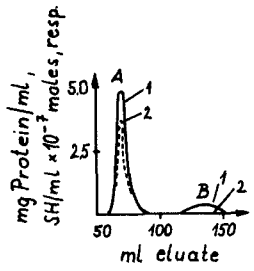

FIGURE IB

Separation of the watersoluble constituents of animal tumor cells (NK-lymphosarcoma, mice) into protein-(A) and non-protein-(B)-thiols by sephadex G25, fine.[22] Curve 1: SH-concentration; curve 2: Protein concentration, in the eluate.

SH-groups. This can be achieved as well by exogenic attacks (administration of SH-reagents or X-rays etc.) as endogenically by α-ketoaldehydes which possess high reactivity to SH-groups and function according to a hypothesis of Szent-Györgyi[21a] and Együd[21b] as a biogenic brake for cell division. The action of those biogenic growth inhibitors is controlled by the glyoxalase system which converts α-ketoaldehydes into the biologically much less reactive α-hydroxy-carboxylic acids.

Finally let us mention the SH-groups of insoluble structural proteins (PSH_S). They belong to scleroproteins which make up the membranes, *e.g.*, of the outer cell wall, ergastoplasm, mitochondria and nucleus. They control the binding of substances to the membranes and their permeation through them as well as the activity of membrane-bound ATP-ases or of other membrane-bound enzymes such as of succinate dehydrogenase or the efficiency of the respiratory chain in the mitochondria. Also the proteins of the mitotic apparatus (spindles, asters) may consist of insoluble fibrous proteins showing the characteristic appearance and disappearance during the different phases of mitosis (*cf.*, Fig. I).

II. METHODS FOR PREPARATIONS AND SH-DETERMINATIONS

As this article should not and cannot be a general survey on SH-compounds, in the following only those methods of quantitative determination will be briefly described which have been employed for our work specifically dealt with here.

A. NPSH and PSH_L (1)[22]

To avoid uncontrolled effects of day time on SH-levels, for all preparations of organs and cells, animals were killed at 10.00 am. Slices of the organs or single tumor cells were investigated either without pre-incubation or after 30 min incubation at 37°C aerobically in Krebs-Ringer-PO_4-buffer at pH 7.4, containing 5×10^{-3} *M* hydroxypentenal (or other reagents) and 0.9% NaCl, respectively, for controls. Thereafter, slices or cells were homogenized 2 min with an ultra-turrax at 0°C under N_2 in isotonic NaCl solution containing 10^{-3} *M* EDTA and 10 volume % 0.15 *M* PO_4-buffer of pH 8. After 30 min centrifuging at 1000 g and 0°C, the slightly opalescent supernate was removed and recentrifuged again for 30 min at 20,000 g. The now clear solution (5 ml) was applied to the top of a sephadex column (G 25 fine) which was flushed before fractionation with the same N_2-saturated medium as used in homogenization. The same medium also was used for fractionation under N_2. This procedure allowed the separation of NPSH from PSH_L as Figure IA shows. The complete operation was tested before with mixtures of bovine serum albumin and CySH, and it was shown that practically 100% of protein- and non-protein thiols could be found in the eluate. In contrast to SH-

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determinations of other authors, this procedure allows the direct and separate determination of NPSH and PSH_L. The eluate fractions containing the PSH_L and NPSH, respectively, were collected and SH were determined with dithionitrobenzenoic acid (DTNB) according to Ellman, protein with biuret.

B. Total SH (= NPSH + PSH_L + PSH_S)[23]

50×10^6 native EATC (6th–8th day post implant) were suspended in 5 ml Krebs-Ringer-PO_4-buffer pH 8 and 0.5 ml DTNB solution 5×10^{-3} *M*, were added and made up to 10 ml with PO_4-buffer. The mixture was homogenized for 2 min, allowed to stand for 30 min and centrifuged 20 min with 10,000 rpm. The extinction of the supernatant was measured compared with a corresponding blank.

C. Cytospectrometric Determinations

Quantitative information on PSH of single cells is undoubtedly an urgent need in cytochemistry which could not be fulfilled until now because no methods were known for direct quantitative determination. Esterbauer[24,25] is credited with having studied very carefully and exactly the reaction between thiols and dihydroxydinaphthyldisulfide (DDD) and the following coupling with different azo-compounds. The DDD-method had been developed by Barrnett and Seligman[26] and still it was assumed that it would be suitable only for qualitative recognition or relative determinations of cellular SH-groups. However, Esterbauer found that DDD reacts stoichiometrically with ethylthioglycolate and isolated and purified the formed azodyes and measured their absorption spectra. After adsorption on a model protein, the absorption changes slightly giving a maximum of 560 nm with a molar extinction coefficient ϵ of 19,000. With this value, Esterbauer determined directly the PSH of different biological samples in macroscopic scale and found excellent agreement with the results of determination with Ellman's reagent. Thereupon, the attempt was made to apply the ϵ-value also for microspectrometric investigations. With EATC we first found excellent coincidence between macroscopical determinations with Ellman's and Barrnett's reagent, respectively.[27] Later, Esterbauer, *et al.*,[28] found that the originally recommended 24 hr incubation in DDD should not be employed generally, because a secondary reaction between DDD and, probably, protein-disulfide groups contributes to a measurable extent when the primary reaction between DDD and protein-SH-groups is coming to end. Because in different cells both reactions run with different rates and extent, it is necessary to prepare with each cell type investigated a special kinetic diagram. With EATC, for example, it was found that after 7 hr incubation in DDD reagent, practically all of the available SH-groups have reacted with DDD and the second reaction still does not make important contributions. After 24 hr incubation, however, contributions due to the secondary reaction can already amount to 25%.

Figure II shows the kinetic diagram with EATC incubated different times in DDD and stained with fast blue B, and the two-step reaction is clearly seen.

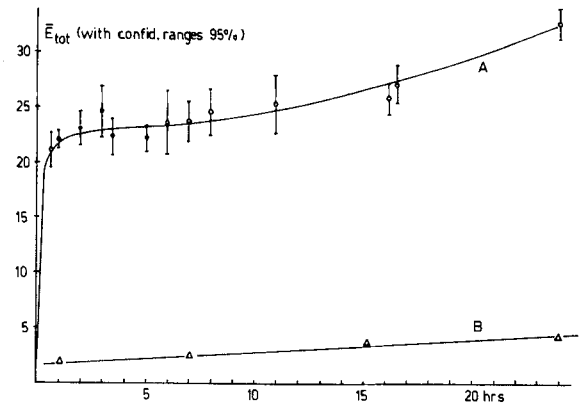
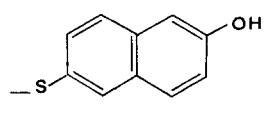


FIGURE II

Mean values E_{tot} at 550 nm with confidential ranges at 95% niveau of EATC after different times of incubation in DDD and stained with fast blue B (curve A). EATC stained with fast blue B, for controls (curve B).[28]

Another problem arises from the correct blank for which the following possibilities are to be envisaged:

a) unspecific adsorption of the DDD
b) reaction of DDD with other groups than SH and –S–S–
c) reaction of azo coupler with other groups than



The relevant experiments of Nöhammer have shown that possibilities a) and b) can be neglected, but that the azo coupler alone is bound to the cells to a certain extent. Therefore the sufficiently correct blank can be determined by treating the cells preincubated with 50% ethanol-veronal 15 min with fast blue B and measuring the extinction at 560 nm.

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III. NPSH IN DEVELOPING DENA-HEPATOMA

As an example for the relationship between NPSH and malignancy, the NPSH content of rat liver was determined during the development of a carcinogen-induced hepatoma in Sprague-Dawley rats. The carcinogen diethylnitrosamine (DENA) was administered with daily drinking water.[29] The following stages were investigated:

Stage 0: Livers of the healthy animals;

Stage 1: 63-68 days after first administration: the livers showed a somewhat pale surface interspersed with numerous minute nodes;

Stage 2: 112-122 days after first administration: the whole liver was filled with massive tumor nodes ranging in size from that of pin heads to that of small cherries.

In each stage, NPSH and PSH_L were determined and the results are illustrated in Figure III. The values for NPSH contents are distributed normally in agreement with the results of Calcutt.[30] The mean value found in the normal livers (5.07 ± 0.22 μmoles/g w.w.) corresponds well to previous data.[22]

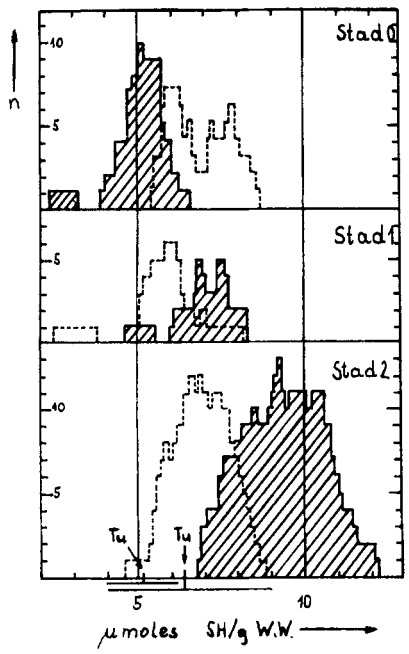

FIGURE III

Statistical distributions of NPSH ▨ and PSH ⬚ in normal rat liver (0) and two stages (1 and 2) of development of DENA-hepatoma.[1,29] n = number of single determinations. Tu = values, with standard deviations of pure tumor tissue; left: PSH_L, right: NPSH.

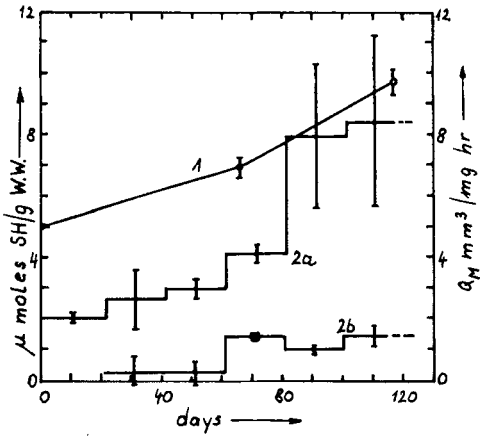

FIGURE IV

Increase of NPSH (curve 1),[29] anaerobic (curve 2a) and aerobic (curve 2b) glycolysis,[37] during development of the DENA-hepatoma.

During the development of the hepatoma, NPSH increase significantly (6.99 ± 0.33 Stage I, 9.82 ± 0.41 Stage 2). Similar effects were observed already with hepatoma induced by other carcinogens, such as aminoazo compounds[31,32] and with skin tumors of mice induced with polycyclic hydrocarbons.[33] Thus, increase in NPSH may be, according to Harington,[34] a more general symptom of tumorigenesis. Yanari[35] and McIlvain[36] pointed out an interesting correlation between the synthesis of glutathione (GSH), the main component of NPSH, and glycolysis stating that only an active glycolysis provides the optimal conditions for a stimulated GSH synthesis. A correlation like this can be observed also with the DENA-hepatoma when comparing the increase in NPSH with glycolytic activities measured by Heise and Görlich;[37] Figure IV demonstrates the close temporal connection, especially to anaerobic glycolytic activity.

At Stage 2, samples of each liver lobe were taken, in case of greater nodes, when possible one half of the node + adjacent liver tissue, avoiding most carefully necrotic parts. Thus, the values obtained could not be ascribed to 100% tumor tissue. In more progressed stages, it was possible to take also pure tumor tissue, avoiding of course, necrotic parts. The NPSH of such tumor tissue was definitively lower than those of Stage 2: 6.43 ± 3.18 μmoles/g w.w.

Another function of NPSH, observed in EATC, will be mentioned later (p. 206): The protection of PSH (and therewith metabolic activities) against the attack of an SH-reactive substance.

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IV. PSH_L IN DIFFERENT ANIMAL TUMORS

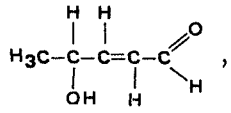
Figure III shows that the PSH_L of normal rat liver follow a two-headed distribution corresponding well to the results of Calcutt.[30] At Stages 1 and 2 in development of DENA hepatoma the values are normally distributed.

At Stage 1, the PSH_L are reaching a significant minimum: 5.76 ± 0.45 μmoles/g w.w. compared with 6.94 ± 0.23 (Stage 0) and 7.17 ± 0.31 (Stage 2). On the basis of the protein determinations in the eluate fractions containing the PSH_L the PSH_L/mg soluble protein are evaluated. It was found that the content of the soluble proteins in Stage 1 and 2 (0.119 ± 0.007 and 0.114 ± 0.005 μmoles/mg protein, respectively) lay about 11 to 16% significantly above the value of normal liver (0.103 ± 0.002). This increase is, however, still very small, and possibly stands in connection with increased proliferative activities of the developing hepatoma. A connection like this could be found much more evidently in faster growing tumors.

For this purpose the NPSH, PSH_L and doubling times (t_d) of the tumors shown in Table II were determined.

These data suggest that tumors with a somewhat higher growth rate possess in general higher SH-contents of their soluble proteins. This assumption is confirmed by plotting the PSH_L values against log t_d as illustrated in Figure V, pointing to a linear regression if, however, with considerable deviations.

The sensitivity of PSH_L to a specific SH-reagent, 4-hydroxy-2,3-transpentenal (HPE)



provides more information on the relationship between PSH_L and tumor malignancy. HPE is the 5-C–homologue of 4-hydroxy-2,3-transalkenals, a hitherto unknown group of aldehydes, the first member of which, 4-hydroxoctenal (HOE) was isolated by Schaunstein *et al.* from autoxydized linoleic acid ethylester.[38] The outstanding biochemical and biological properties of this substance–potent and highly selective inhibitions of certain SH-controlled metabolic steps and of growth of some animal tumors[39]–made it desirable to have it available in larger amounts. This could be achieved by developing syntheses for hydroxyalkenals as done successfully by Esterbauer, *et al.*[40] Thus gram quantities not only of HOE but also of homologous hydroxyalkenals with chain length between 4 and 14 C were available. However, most of the further investigations were carried out with HPE because the relatively good solubility in water of this substance is a considerable advantage for biological work. An information survey on the biochemical effects of HPE will be given later.

All the effects named above are caused by the reaction of HPE with functional SH-groups. At an equimolar ratio or with a surplus of HPE, the mech-

TABLE II

PSH_L and doubling time of different animal tumors. The PSH_L were determined after 30 min preincubation of single cells and solid tumor slices, respectively, in 0.9% NaCl

Days p implant.	Tumor	PSH_L (μM/mg Prot.)	t_d (days)
6–8	Ehrlich, Heidelberg-Lettré, Ascites, mice	0.126 ± 0.002	2.0 ± *ca.* 0.3
	Ehrlich, London, „ „	0.150 ± 0.004	1.9 ± *ca.* 0.3
	NK-Lymphosarcoma, „ „	0.121 ± 0.003	2.5 ± *ca.* 0.3
	Sarcoma 180, „ „	0.127 ± 0.006	2.0 ± *ca.* 0.3
50–57	HP-Melanoma, solid, „	0.062 ± 0.003	7–8
11–15	Ehrlich H.-L., „ „	0.072 ± 0.009	5.0 ± *ca.* 1
	NK-Lymphosarcoma, „ „	0.090 ± 0.005	5.2 ± *ca.* 1
	Sarcoma 180, „ „	0.118 ± 0.012	4.0 ± *ca.* 1
14–15	Yoshida Sarcoma, „ rat	0.116 ± 0.005	6.5
14–15	Rhabdomyosarcoma, „ „	0.072 ± 0.008	7
ca. 120	DENA Hepatoma (pure tumor) „ „	0.084 ± 0.004	*ca.* 100

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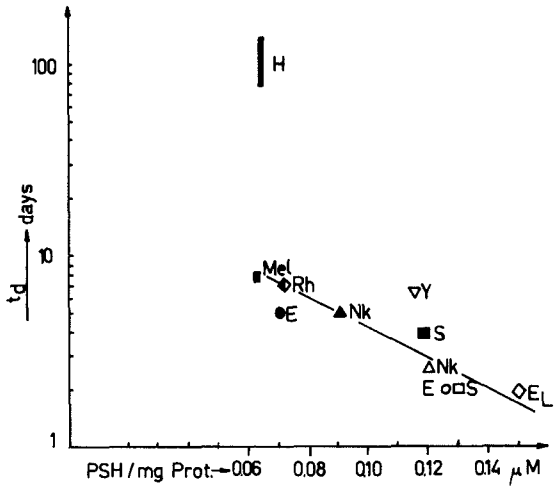
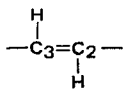


FIGURE V

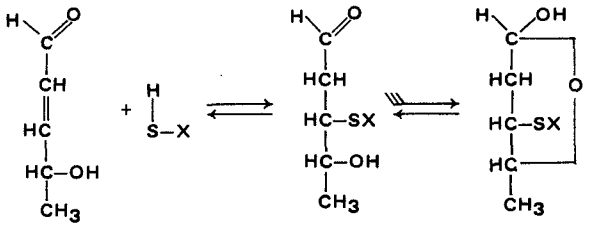
SH-content of soluble cell proteins of animal tumors with different growth rate as a function of the doubling time t_d.

	Tumor		Ascites	Solid
E	Ehrlich, Heidelberg-Lettré	Mice	○	●
E_L	Ehrlich, London	Mice	◇	
S	Sarcoma 180	Mice	□	■
NK	Nemeth-Keller, lymphosarcoma	Mice	△	▲
Rh	Rhabdomyoscarcoma	Rat		◆
Mel	Melanoma, Harding-Passey	Mice		▮
H	DENA-hepatoma	Rat		▮

anism of this most important reaction consists of an addition of one mole SH to the



group of HPE under formation of a stable thioether adduct followed by a subsequent cyclization of the formed saturated aldehyde to a furanoid ring:



This reaction mechanism has been elucidated with glutathione as model substance by Schauenstein *et al.*[41] and later by Esterbauer.[41a] On the basis of these facts, it is understandable that after incubation of living cells with HPE, drastic decreases of PSH_L and NPSH can be found.

In Figure VI the percentual losses of PSH_L (in terms of μmoles SH/mg soluble protein) are plotted against log t_d. As before with SH/mg protein, a

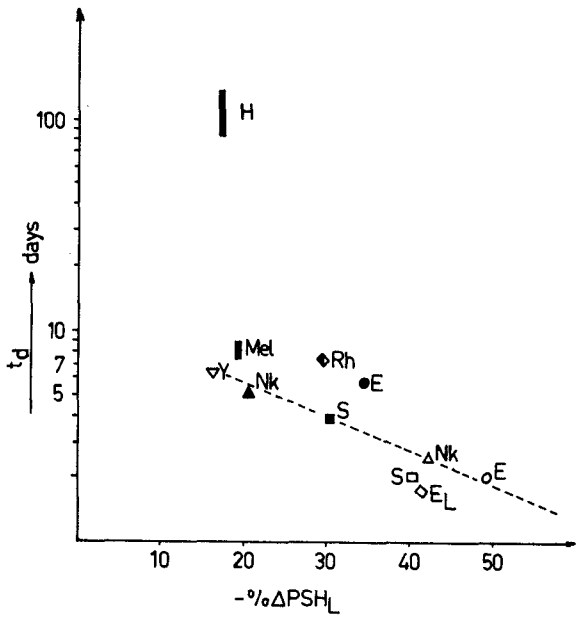


FIGURE VI

Percental loss of SH-groups in soluble proteins of animal tumors after incubation in 5 x 10^{-3} *M* HPE (controls: in 0.9% NaCl), as a function of doubling time t_d (1). Symbols corresponding to Figure V.

linear relationship can be seen, the deviation of the single points being already considerably smaller. Almost the ideal linear function is observed if the absolute SH losses of the soluble proteins (−ΔSH (μm)/mg protein) are plotted against the log t_d, as Figure VII clearly demonstrates. This suggests that

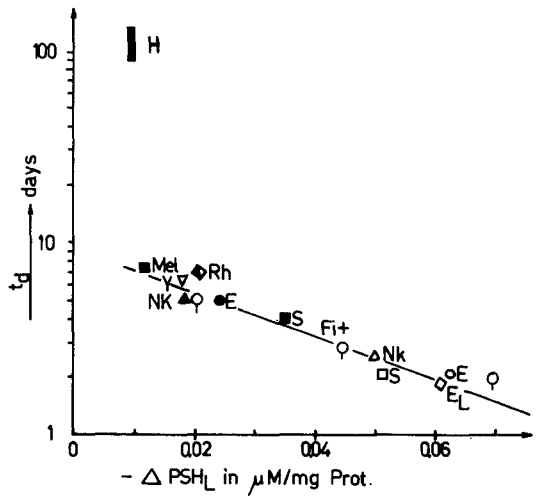


FIGURE VII

Absolute loss of SH-groups in soluble proteins of animal tumors and chicken fibroblasts (+Fi) after incubation in 5 x 10^{-3} *M* HPE (controls: in 0.9% NaCl), as a function of the doubling time t_d. (1). ♀: PSH_L increments calculated on the basis of the straight line in Figure V, other symbols corresponding to Figure V.

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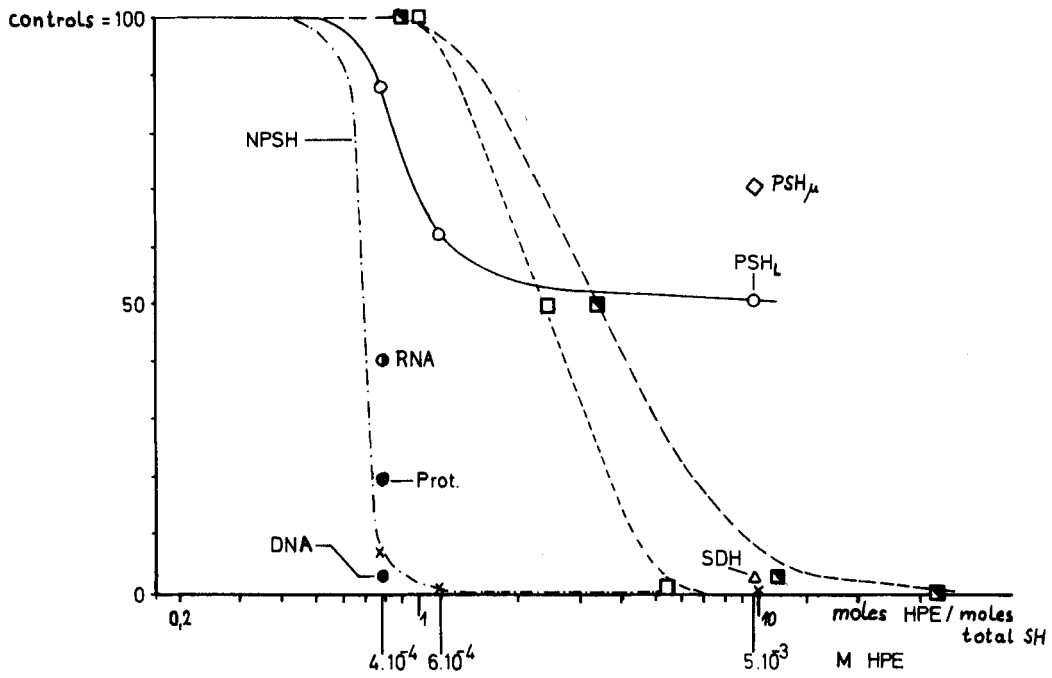

FIGURE VIII

Dosis-response-curves for HPE with EATC. Abscissa: Upper scale: Ratio moles HPE in the incubation medium/moles of total SH of cells (= NPSH + PSH$_L$ + PSH). Lower scale: Molar concentration of HPE in the incubation medium. Ordinate. Relative decreases of NPSH (moles/10^6 cells), PSH$_L$ (moles/mg protein), PSH$_\mu$ (10^{-14} moles/single cell), and some SH-controlled cell functions.

×: NPSH;[1] ◑: RNA-synthesis;[42] □: anaerobical glycolysis;[1]
○: PSH$_L$;[1] ●: DNA-synthesis;[42] ◇: microscopically determined PSH$_\mu$;[27,28]
⊕: Protein synthesis;[42] ◪: O_2-uptake;[1] △: succinate-dehydrogenase-activity.[43]

among the bulk of SH-groups of the soluble cell proteins there must exist certain thiols with which HPE reacts selectively. The number of these HPE sensitive protein thiols stands apparently in a distinct relation with the doubling time according to

$$t_d = e^{A - B(SH)},$$

where (SH) is the number of HPE sensitive SH-groups/mg prot., A and B are constants just being evaluated together with J. Gölles*.

Hence, we can see that the investigated cells with t_d shorter than *ca.* 9 days contain the more SH-groups in their soluble proteins the faster they divide (Figure V). Trying to fit something like an ideal straight line through the single points of Figure V, we can see that the tumors with t_d 7–8 days will have an average of 0.07 μmoles SH/mg protein, whereas cells with approximately 5, 3 and 2 days of t_d have 0.09, 0.12, and 0.14 μmoles SH/mg protein, respectively.

Thus the differences to the SH-content of 0.07 amount to *ca.* 0.02 for cells with t_d of *ca.* 5 days, 0.05 for cells with t_d of *ca.* 3 days, 0.07 for cells with t_d of *ca.* 2 days.

The points with symbol ⚲ in Figure VII show that these differences, that means the respective increases of SH-content of the soluble proteins, correspond surprisingly well to the losses of PSH$_L$ brought about by incubation in 5 x 10^{-3} *M* HPE. Thus we can conclude that HPE reacts with high selectivity with the respective increment of PSH$_L$ which is apparently of functional importance for cell division. The point with the symbol (+Fi) in Figure VII indicates that this principle seems to be of some universality and not restricted to malignant cells.

The question arises: to what kind of protein do these SH-groups belong? Figure VIII gives first information for the example of EATC. The diagram shows dosis-effect-curves for HPE concerning different parameters. On the ordinate, relative inhibitions are plotted, 100% corresponding to controls.

The abscissa shows two different scales: The lower gives 3 different molar concentrations of HPE in the incubation medium; as the volume of the incubation medium is known, the moles of HPE acting on the

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given cell number is equally known, of course. Furthermore, as the average amount of total SH (NPSH + all of PSH) are known for the EATC, each HPE concentration in the medium corresponds to a certain ratio $\frac{\text{moles HPE}}{\text{moles SH}}$ and these ratios are scaled logarithmically on the upper abcissa axis.

The diagram demonstrates the different sensitivity of different parameters to HPE: one of them is the biosynthesis of DNA as measured by Bickis *et al.*[42] It can be seen from the diagram that DNA-biosynthesis is already inhibited to 96% when the loss of PSH_L comes to *ca.* 10% at 4.10^{-4} *M* HPE. Hence we can say that at 5.10^{-3} *M* HPE, where the loss of PSH_L reaches its final amount, a part of the HPE-blocked PSH belongs to the proteins of the DNA synthetase system which is in fact known to be well soluble and to contain functional SH-groups.[8a]

As the points "RNA" and "Prot" indicate also the SH-groups of RNA and protein biosynthetases are involved.

Summarizing we can assume that approximately one fifth to one-fourth of the blocked PSH_L probably belong to the proteins of nucleic acids and protein synthetase systems. As at 5×10^{-3} *M* HPE O_2-uptake and anaerobic glycolysis have been inhibited to practically 100%, another part of the HPE-blocked SH-groups belong to soluble enzymes of energy metabolism as could be experimentally proved with glyceraldehydephosphate-, lactate-, isocitrate and malate dehydrogenases[42,43] and parts of the respiratory chain[44]. However, an essential part of the blocked PSH_L must belong to soluble proteins being of direct importance for tumor growth as stated above. It seems very likely that these are the acidic residual proteins being important stimulators for DNA polymerase activity and rich in SH-groups.[8-18,45]

It was shown (p. 205, Fig. VII) that Ehrlich ascites tumor cells after 30 min. pretreatment with 5×10^{-3} *M* HPE lose about 0.06 μmol SH per mg of soluble proteins. In this connection it appears noteworthy that Modig[45a] found that DNO-bound proteins from these cells contain 0.059 μmol SH per mg protein.

Figure VIII demonstrates furthermore that already at 4×10^{-4} *M* HPE the NPSH have decreased down to 7% and are completely consumed at 5 and 6×10^{-4} *M* HPE, whereas the PSH_L do not show any significant decrease at 4×10^{-4} *M* HPE. This indicates that in EATC the PSH_L do not show any significant decrease at 4×10^{-4} *M* HPE. This indicates that in EATC the PSH_L begin to react with HPE only after the bulk of the NPSH has already been consumed. The narrow range between the NPSH- and PSH_L-decrease suggests a stepwise reaction of SH systems with HPE pointing to a protecting effect of the NPSH for the greater part of the HPE sensitive PSH_L against the attack of the reagent. An effect like this seems to be explainable considering that NPSH—predominantly GSH—are homogeneously dispersed in the cytoplasm and much more readily accessible to HPE than the complicated folded protein molecules.

The observation that after incubation with 5×10^{-3} *M* HPE the NPSH disappear completely whereas PSH_L only in part was made with all the tumors named in Table II and also with normal rat kidney, jejunum, diaphragm; in liver, with the highest NPSH content no PSH_L decrease was found and the NPSH decrease only by 45%.[22]

These observations demonstrate apparently the general protecting effect of the NPSH for PSH not only against the attack of SH reactive agents but probably also against X-rays which, as is well known, also attack the SH group.

V. MICROSPECTROMETRICALLY DETERMINABLE PROTEIN–SH

Several staining methods exist for making protein SH-groups visible for qualitative or at the most relatively comparing cyto- and histochemical studies. As stated above (p. 202), the direct and absolute quantitative information only has been attainable on the basis of the work together with Esterbauer[24,25,27] and Nöhammer[28] with the DDD-fast blue B method of Barrnett and Seligman.[26]

Thus, quantitative information about the content of single cells of SH-groups as well in the cytoplasm as in the nucleus are now available. Let us take the EATC for an example.

The cells had been taken immediately from the freshly killed animal 6 to 8 days post implant. After fixation in alcohol they were treated following the prescriptions worked out by Esterbauer and Nöhammer 7 hr with DDD and stained with fast blue B. After adequate washing they were prepared for cytospectrometric measurement.

Each single cell was scanned and the total extinction at 560 nm was read from the digital output. Another aliquot of cells was directly stained with fast blue B (without DDD pretreatment) and their extinction was measured at 560 nm (Table III).

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TABLE III

$\bar{E}_{tot}$-values at 560 nm of EATC, treated with DDD and fast blue according to Barrnett and Seligman,[26] Esterbauer,[27,28] Nöhammer[48] and Weber.[46] n = number of measured cells; $\bar{E}_{tot}$ = mean value of total extinction; S = standard deviation; Cf 95 = confidential range at 95% level.

Treatment of the cells	n	$\bar{E}_{tot}$	S	Cf 95
DDD-fast blue	50	23.73	± 6.87	± 1.91
Fast blue only	50	3.45	± 1.34	± 0.37
		20.28		± 1.95

Applying the correct molar extinction coefficient determined by Esterbauer[27] of 19,000, we obtain for the single an average of

$$1.07 \pm 0.10 \times 10^{-14} \text{ moles SH.}$$

This result was established by measurements of a total of 400 single cells[46] and by measurements on a preparative scale indicating a number of 1.5×10^{-14} moles SH/single cell.[46]

The first question we shall consider now, is what types of substances these microscopically determined SH-groups may belong to and where are they mainly located?

The fact that several thorough washings are involved in the preparation suggests that the NPSH were removed almost quantitatively and therefore should not be included in the microspectrometric value. As far as the PSH_L are concerned, it can be assumed that the major part of the soluble proteins are getting insoluble by a fixation process and thus the major part of PSH_L may be registered microspectrometrically.

Finally, there exist several proteins in the cell which are the essential components of structural elements such as outer cell wall, membranes of the mitochondria, the ergastoplasm, ribosomes, nucleus, etc. These proteins are insoluble and their SH-groups (PSH_S) are certainly registered totally by the microspectrometer. If we call the microscopically measurable SH-groups PSH_μ, we can take the following relation as probably correct in first approximation:

$$PSH_\mu \simeq f \,.\, PSH_L + PSH_S$$

In view of the factor f and the value of PSH_S the following considerations can be made: Hofer[23] determined macroscopically the total SH-content of EATC to $\sim 1.9 \times 10^{-14}$ moles/cell. According to Schindler[1] the NPSH of non-preincubated EATC amount to $0.27 \pm 0.05 \times 10^{-14}$ moles, the PSH_L to $1.11 \pm 0.17 \times 10^{-14}$ moles; hence for the PSH_S result a difference of 0.52×10^{-14} moles/single cell. This is in good accordance with the statement of Jocelyn[47] that in general the PSH_L make the bulk of the PSH.

As the PSH_μ were determined to 1.07×10^{-14}, comprising certainly all of the PSH_S, it follows for the microscopically determinable PSH_L an amount of 0.55×10^{-14} moles that is ca. 50% of the total PSH_L.

Let us now consider what special advantages and information may be derived from PSH_μ-measurements:

1) The often very valuable possibility of obtaining quantitative information already from minimal quantities of biological material. Statistical evaluations of our measurements show unequivocally that mean E_{tot}-values obtained only from at least 50 single cells are already in good approximation to mean values obtained from greater cell numbers, and they will not change significantly if we compare 100, 200 and several hundred cells. Therefore, it is not necessary to investigate 100,000 cells as postulated now and then.

2) The fascinating possibility to reveal the main sites of PSH within the cell. As an example can serve the micrographs of EATC and normal rat liver cells in Figure IX after staining with DDD-fast blue photographed at 560 nm. EATC, as rapidly dividing cells, have extremely great nuclei filling out nearly the total volume of the cell and it can be seen from the photo that the 1.07×10^{-14} moles PSH_μ found in the EATC stem practically entirely from nuclear proteins pointing to their belonging to NA synthetase systems and DNA bound residual proteins. On the contrary, the normal liver cell shows a considerably reduced proportion of the cell occupied by the nucleus. The photo demonstrates that the major parts of PSH_μ [$5.07 \pm 0.30 \times 10^{-14}$ moles[46]] are located in the cytoplasm indicating their predominant association with cytoplasmic proteins, above all enzyme proteins, being of high activities in the liver cytoplasm as it is widely known.

3) The statistical evaluation of the single values enables insights into the properties and behavior of the single cells of a given collective, detection of sub-groups, interpretation of obtained mean values, etc. This shall be illustrated again with EATC. Figure X shows statistical distribution diagrams of E_{tot}-values of DDD-fast blue stained EATC as well after incubation in 0.9% NaCl as in 5×10^{-3} *M* HPE. The E_{tot}-values of NaCl incubated cells are slightly right-handed asymmetrically distributed suggesting the presence of sub-groups of cells with higher PSH_μ contents. After incubation in HPE, a general loss also of PSH_μ was to be expected on the basis of the foregoing considerations and experimental results (*cf.* pp. 204-5). The decrease of PSH_μ could actually be registered

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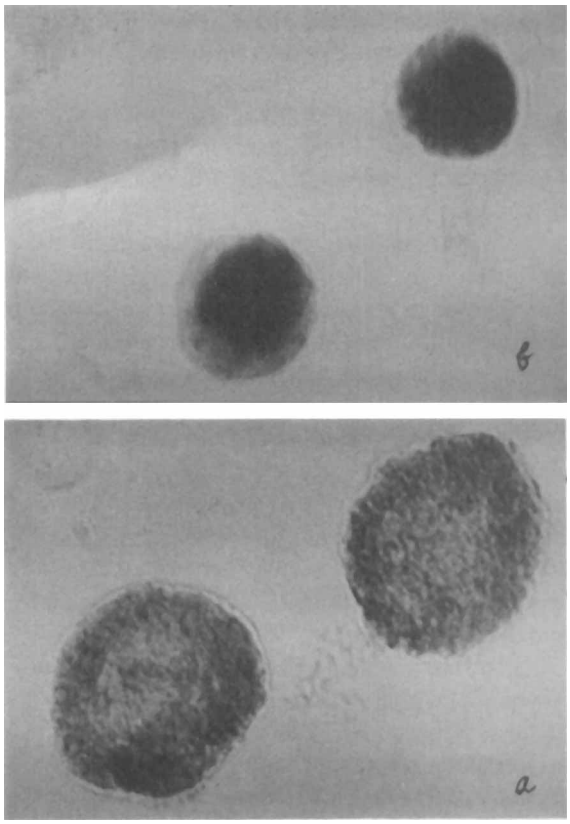

FIGURE IX

Micrographs taken at 560 nm of DDD-fast blue B-stained cells from: a) normal rat liver; b) Ehrlich ascites tumor of mice.[46]

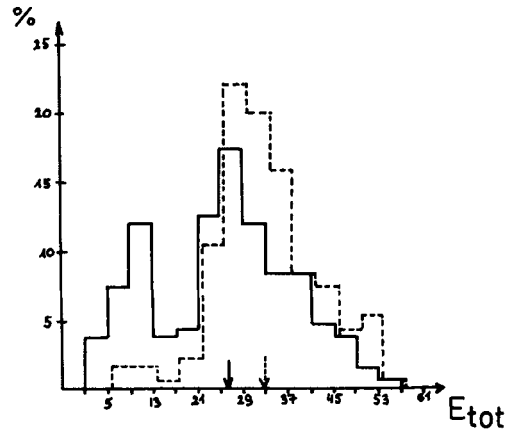

FIGURE X

Statistical distributions of E_{tot}-values taken at 560 nm from EATC stained with DDD-fast blue B.

--------: After 30 min. incubation in 0.9% NaCl at 37° C;
————: After 30 min. incubation in 5×10^{-3} *M* HPE at 37° C
} 191 cells measured.[28,46]

as $\bar{E}_{tot}$ for NaCl-incubated cells lies at 30.15 ± 9.9 (s), for HPE-incubated cells at 21.48 ± 11.18 (s). The difference of 8.67 corresponds to a loss of 0.46×10^{-14} moles PSH_μ per single cell; the loss occurs with 99% probability within a range of confidence of ±0.12 and thus is highly significant.[48] Only the statistical distribution diagram reveals how the shift of the mean E_{tot}-values is realized. After HPE incubation the distribution diagram loses *ca.* 40% of its area corresponding to cells with E_{tot} values $\geqq \bar{E}_{tot}$ and a corresponding new peak appears at very low E_{tot}-values (around ~8). Thus the shift of the E_{tot} from 30.2 to 21.5 is brought about by a clearly different reaction of the PSH_μ or the different cell types being present in the investigated collective. However, it cannot be decided whether 60% of the cells have HPE-resistant PSH_μ or whether the PSH_μ of each single cell have reacted with HPE but to widely varying extents. This still unsolved problem is being investigated; preliminar results suggest that the PSH_μ of each cell react with HPE but that extremely strong reaction occurs without exception in cells with high PSH_μ-densities.[48] Statistical evaluation of PSH_μ-values of single cells furthermore provides the possibility of differentiating between tumor and normal cells, at a microscopic scale. Such investigations were undertaken by Tolmashev[48a] who found that cells from normal epithe lium of human cervix uteri give extinction values normally distributed around a sharp maximum corresponding to a relative absorption unit of unity. Cells from carcinoma of the cervix uteri gave a righthanded asymmetrical distribution diagram with a broad maximum at 3 relative absorption units indicating the presence of single cells with PSH_μ content many times greater. Our own investigations[48b] confirmed these res in an absolute scale but extended them additionally in a fascinating way. It was found that not only the true tumor cells have increased PSH_μ-values but that also the cells of the upper layers of the cervix epitheliu which appear to be morphologically intact have significantly increased PSH_μ-values if taken from cases suffering from carcinoma of the cervix uteri. Apparent also some diagnostic aspects seem to be given herewith

VI. ACTION OF DIFFERENT SH-REAGENTS ON ^{3}H-THYMIDINE INPUT IN EATC AND ON GENERAL TOXICITY TO MICE

In the foregoing we dealt with the different types of SH-compounds of the living cell and with the

The action of a given SH-reagent on a given thiol is induced by the chemical reactivities of both com-

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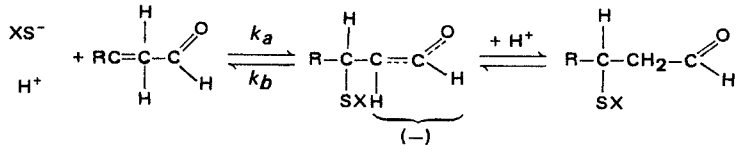
of the reaction between both substances. It is the merit of Esterbauer having elucidated these kinetics of the reaction of α,β-unsaturated aldehydes with model thiols very carefully and extensively,[49] after this problem had been studied before by many authors, especially by Friedman *et al.*[50] and Cavins.[51] According to Esterbauer's work,[49] the actual reactant is the sulfhydryl anion which adds to the C_3=C_2-group being in a mesomeric resonance with the vicinal carbonyl group:



Depending on the pH, the rate controlling step is either the addition of XS^- or the stabilization of the mesomeric intermediate of the adduct by H_3O^+ or H_2O. Thus, the reaction is catalyzed predominantly by OH^- but also by acids among which buffer acids such as $H_2PO_4^-$ or HPO_4^{--} are of greatest significance.

The effect of buffers varies widely for different reactions of a given α,β-unsaturated carbonyl with different thiols. For example, the reaction with CySH is practically not accelerated, with glutathione moderately and with thioglycolic acid esters strongly.

It should be emphasized that the chemical structure of the α,β-unsaturated carbonyl compound greatly influences its reactivity to a given thiol. As a quantitative measure the rate constant k_1 can serve. The rate of formation of the addition product between a given thiol RSH and an aldehyde is given by

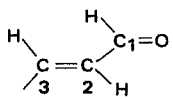
$$v = k_1\,[\text{aldehyde}]\,.\,[RSH + RS^-];$$

k_1 could be calculated from

$$k_1 = k_a\,.\,\alpha_{SH}\,.\,\beta\,(M^{-1}.\sec^{-1}),$$

where k_a and k_b are the rate constants for the formation and redissociation of the mesomeric addition intermediate, α_{SH} the electrolytic dissociation of RSH and β a parameter for the proton transfer rate from proton donors (such as H_2O, H_3O^+, buffer acids) to the mesomeric intermediate.

Together with Esterbauer and Zollner[52] and Kollaritsch,[53] a series of α,β-unsaturated carbonyl compounds was studied with respect to reactivity to GSH in M/15 phosphate buffer pH 7.4 at 20°C, general toxicity and inhibition of ^{3}H thymidine input into EATC cells were selected in order to study the influences of the following variations to the basic molecule:



a) affixing an aliphatic chain to C_3
b) introducing a hydroxy group } vicinal to C_3
c) introducing a carbonyl group }
d) substitution of aldehyde H by $-CH_3$.

TABLE IV

Reactivity of different SH-reagents to glutathione (column 3), inhibition of ^{3}H-thymidine input into EATC (column 2) and general toxicity to mice (column 4): column 2: half-maximal dosis (moles/10^6 cells); column 3: rate constant for formation of primary addition product; column 4: LD_{50} (moles/kg body weight) 24 hours after single intraperitoneal injection.

Substance	Inhibition thymidine-input	k_1	LD_{50}
2-Propenal	0.25×10^{-8}	121	1.03×10^{-4}
2-Butenal	5.6×10^{-8}	0.78	23.0×10^{-4}
2-Pentenal	17.5×10^{-8}	0.47	29.0×10^{-4}
2-Hexenal	8.75×10^{-8}	0.33	23.8×10^{-4}
4-OH-5C-enal	4.8×10^{-8}	2.19	9.9×10^{-4}
4-OH-6C-enal	8.1×10^{-8}	1.56	9.8×10^{-4}
4-OH-7C-enal	5.6×10^{-8}	1.83	12.2×10^{-4}
4-OH-8C-enal	4.6×10^{-8}	1.74	14.6×10^{-4}
4-OH-9C-enal	2.5×10^{-8}	1.9	4.4×10^{-4}
4-OH-11C-enal	1.2×10^{-8}	1.47	4.6×10^{-4}
4-OH-14C-enal	1.75×10^{-8}	3.16	
4-keto-5C-enal	0.66×10^{-8}		1.56×10^{-4}
Methylvinyl-ketone	0.5×10^{-8}	31.9	10.8×10^{-4}

The results indicate:

1) The ^{3}H-thymidine-input—a widely known SH controlled system—is inhibited most strongly by acroleine; variation a) lowers the inhibitory effect by the 20-, 30- and 70-fold. Variation b) also lowers the inhibitory effect but not so strongly and depending on the length of the adjacent chain: with 4-, 7- and

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the 6-membered chain 30-fold lower compared with acroleine; with longer side-chains (9- to 14C-homologue hydroxyalkenals) the inhibition is only 4- to 10-fold lower. 4-hydroxypentenal is nearly 4 times more active than pentenal; with the corresponding hexenals practically no difference occurs because 4-hydroxy hexenal shows the lowest inhibitory effect among all investigated hydroxyalkenals.

Variations c) and d) cause inhibitions approximately equal to that of acroleine.

2) The reactivity to GSH runs approximately parallel with inhibitory effects.

3) The same holds for the general toxicity. Thus the results concerning the inhibitory effect confirm and emphasize well that ^{3}H-thymidine-input in general involves SH-groups of functional importance. It is known that ^{3}H-thymidine-input is a rather ambiguous phenomenon involving several processes but especially with HPE it could be proven that in fact the biosynthesis of DNA is concerned.[42,54]

The results concerning the general toxicity show for the first time that also this effect is apparently mediated by attack to vitally functional SH-groups. This surprising result is convincingly confirmed by plotting k_1-values against toxicities in double logarithmic scale (Figure XI). Here again, the question arises as to what substances these SH-groups may belong and where they may be situated in the body.

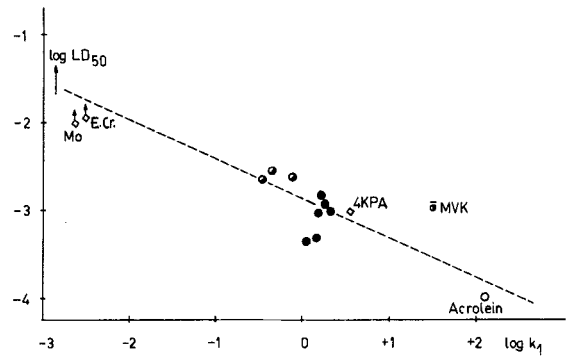

FIGURE XI

General toxicity (mice, single doses, intraperitoneally) as a function of SH-reactivity of some α,β-unsaturated carbonyl-compounds.[52,53] Abscissa: k_1 = rate constant for the formation of intermediate addition product from glutathione and the carbonyls.

Mo : Mesithyloxide; E. Cr. : Ethylcrotonal } Arrows symbolize LD_{50} probably greater than indicated here!
◑ : 2-alkenals.
● : 4-hydroxy-2-alkenals; 4-KPA: 4-ketopentenoic acid } (*cf.* Table IV)
MVK : Methylvinylketone.

However embarrassing this question may appear, some first indications can be given. The first points to the erythrocytes which in their membrane and in the hemoglobin contain a lot of functionally important PSH and are attacked strongly by SH-reagents. Thus damage to the erythrocytes necessarily will cause damage to their vital function as oxygen carriers.

A second indication points to the liver: As we have seen before (p. 206)[22,29] SH-reagents may cause drastic fall in the NPSH, predominantly GSH, which is consumed for transforming α,β-unsaturated carbonyl compounds into substances which are suitable for renal excretion, as already outlined on p. 199. The group of enzymes concerned have been studied thoroughly by Boyland *et al.*[55] They are called glutathione-S-alkenetransferases catalyzing the addition of glutathione-SH to the C=C-group of a series of compounds. Also in the special case of the 4-hydroxy-α,β-unsaturated carbonyls treated here, Esterbauer *et al.*[4] could elucidate in detail the mechanism of detoxification in the liver and renal excretion. He administered to normal rats (250 g.b.w.) 4,5-^{14}C-labeled 4-hydroxypentenal diethylacetal (120 mg/kg b.w.). 24 hr later, 62% of the activity appeared in the urine as 4-keto-3-S-N-acetylcysteine revealing thus the following metabolic steps:

1) Oxidation of the 4-hydroxy group making the C_3=C_2-group *ca.* 7 times more reactive to the addition of glutathione-SH-group as we have seen from Table IV.

2) Addition of GSH on to C_3=C_2-group.

3) Splitting off both of the glycine and glutaminic acid residues from GSH and

4) acetylation of α-NH_2 of the added CySH residue. The steps certainly occur in the liver, the first catalyzed probably by a NADP-dependent oxydoreductase,[56] the second by Boyland's GSH–S-alkenetransferase, the third by glutathionase and the N-acetylation mediated by acetyl-CoA. According to initial results obtained by Esterbauer (still unpublished), a similar mechanism of metabolism is true also for 4-hydroxypentenal. It seems to be very plausible that these reactions cause a severe interference in GSH-metabolism of the liver, quite apart from possible attacks to other SH-functional enzymes.

Hence, first indications could be given for an explanation of the toxicity of SH-reagents on living organism. Let us finally point to the top position of acroleine as to toxicity and SH-reactivity and remember that from this substance arise many of the most serious environmental and air pollution problems.

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References

1. R. Schindler, Thesis, University of Graz, 1971.
2. G. Calcutt, *Brit. J. Cancer*, **19**, 883 (1966).
3. D. Mazia, in "Biolog. Struct. and Function," II, Goodwin and Lindberg, Eds., New York, Academic Press, 1961, p. 475.
4. H. Esterbauer, N. Scholz, and H. Sterk, *Mh. Chem.*, **103**, 1453 (1972).
5. Cf. "Glutathione," Academic Press, 1954, p. 271.
6. Cf. "Glutathione," Academic Press, 1954, p. 165, 183.
7. Glutathione, Proc. 16th Conf. German Soc. Biol. Chem. 1973, G. Thieme, Publ. Stuttgart.
8. H. Busch, Ed., "Molecular Biol. of Cancer," Academic Press, 1974, p. 503.

8. *a.* J. Rossignol, J. Abadiebat, J. Tillit, and A. Recondo, *Biochimie* (Paris) **54**, 319 (1972).

9. H. Sakai and K. Dan, *Exptl. Cell Res.*, **16**, 24 (1959); E. Neufeld and D. Mazia, *Exptl. Cell Res.*, **13**, 622 (1957).

9. *a.* H. Sakai, *Internatl. Rev. Cytol.*, **23**, 89 (1968)

9. *b.* M. Gronow and F. Lewis, *Exptl. Cell Res.* **93**, 225 (1975).

10. J. Hilton and L. Stocken, *Biochem. J.*, **100**, 21C (1966).
11. T. Wang, *Proc. Natl. Acad. Sci., USA*, **54**, 800 (1965).
12. W. Sandritter and A. Kryger, *Z. Krebsforsch.*, **62**, 596 (1959).
13. D. Mazia in "The Cell, III," Brachet and Mirsky, Eds., Academic Press, 1961, p. 77, 251.
14. M. Ord and L. Stocken, *Biochem. J.*, **109**, 24P (1969); *Biochem. J.*, **116**, 415 (1970).
15. S. Panyin, R. Chalkley, S. Spiker, and D. Oliver, *BiBiA.*, **214**, 216 (1970).

15. *a.* M. Lea, M. Koch, and H. P. Morris, *Cancer Res.*, **35**, 1693 (1975).

15. *b.* M. Gronow and T. Tackrah, *Chem. Biol. Interact.*, **9**, 225 (1971).

15. *c.* G. Stein, W. Criss, and H. P. Morris, *Life Sci.*, **14**, 95 (1974).

16. E. Kedrova and W. Rodionow, *Int. J. Radiat. Biol.*, **16**, 359 (1969).
17. M. Ord and L. Stocken, *Biochem. J.*, **112**, 81 (1969).
18. A. Mirsky and S. Osawa, in "The Cell," J. Brachet and A. Mirsky, II, Eds., Academic Press, 1961, p. 677.
19. H. Busch, in "Histones and Other Nucleus Proteins," Academic Press, 1965.
20. G. Calcutt and T. Connors, *Biochem. Pharmacol.*,

21. *a.* A. Szent-Györgyi, Introduction to Subcellular Biology, Academic Press, 1960, p. 45.

21. *b.* L. Együd, *Proc. Natl. Acad. Sci. USA*, **54**, 200 (1965).

22. R. Rindler and E. Schauenstein, *Z. Naturforsch.*, **25b**, 739 (1970).
23. E. Hofer, Thesis, University of Graz, 1975.
24. H. Esterbauer, *Acta Histochem.*, **42**, 351 (1972).
25. H. Esterbauer, *Acta Histochem.*, **47**, 94 (1973).
26. J. Barrnett and A. Seligman, *Science*, **116**, 323 (1952).
27. H. Esterbauer, G. Nöhammer, E. Schauenstein, and P. Weber, *Acta Histochem.*, **47**, 106 (1973).
28. H. Esterbauer, G. Nöhammer, E. Schauenstein, and P. Weber, *Acta Histochem.*, (1975) (in press).
29. E. Schauenstein, R. Rindler, R. Schindler, M. Taufer, and G. Ruhenstroth-Bauer, *Z. Naturforsch*, **26b**, 788 (1971).
30. G. Calcutt, *Brit. J. Cancer*, **18**, 197 (1964).
31. J. Dijkstra, *Brit. J. Cancer*, **18**, 608 (1964).
32. J. Dijkstra and W. Pepler, *Brit. J. Cancer* **18**, 618 (1964).
33. J. S. Harington, *Advances Cancer Res.*, **10**, 247 (1967).
34. J. S. Harington, *Med. Proc.*, **13**, 574 (1967).
35. S. Yanari, J. E. Snoke and K. Bloch, *J. Biol. Chem.*, **201**, 561 (1953).
36. H. McIlwain, in "Glutathione", Biochem. Soc. Sympos. No. 17, Ed. M. Crook, Univ. Press, Cambridge 1959, p. 66.
37. E. Heise and M. Görlich, *Exptl. Cell Res.*, **33**, 289 (1964); *J. Nat. Cancer Inst.*, **35**, 412 (1966).
38. E. Schauenstein, H. Esterbauer, G. Jaag, and M. Taufer, *Mh. Chem.*, **95**, 180 (1964).
39. E. Schauenstein, *J. Lipid Res.*, **8**, 417 (1967).
40. H. Esterbauer and W. Weger, *Mh. Chem.*, **98**, 1884, 1994 (1967).
41. E. Schauenstein, F. Dorner, and J. Sonnenbichler, *Z. Naturforsch.*, **23b**, 316 (1967).

41. *a.* H. Esterbauer, A. Ertl, and N. Scholz, *Tetrahedron*, **32**, 285 (1976).

42. I. J. Bickis, E. Schauenstein, and M. Taufer, *Mh. Chem.*, **100**, 1077 (1969).
43. E. Schauenstein and E. Kapfer, *Mh. Chem.*, **103**, 1200 (1972).
44. E. Schauenstein, H. Verdino, and M. Taufer, *Mh. Chem.*, **101**, 1180 (1970).
45. F. Knock, "Anticancer Agents", Ch. Thomas, Publ., USA, 1967.

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46. P. Weber, Thesis, University of Graz, 1974.
47. P. C. Jocelyn, "Biochem. of the SH-group", Academic Press, 1972.
48. G. Nöhammer, E. Schauenstein, and P. Weber, *Acta Histochim.*, (1975) (in press).
48. *a.* W. Tolmashev, *Vopr. Onkol.*, **20**, 17 (1974).
48. *b.* F. Bajardi, G. Nöhammer, E. Schauenstein, and Cl. Unger-Ullmann, 2nd World Congr. Cervixpathol., Graz, 1975. Cl. Unger-Ullmann, Thesis, University of Graz, 1976.
49. H. Esterbauer, *Mh. Chem.*, **101**, 782 (1972).
49. *a.* H. Esterbauer, H. Zollner, and N. Scholz, *Z. Naturforsch.*, **30c**, 466 (1975).
50. M. Friedman and J. S. Wall, *J. Am. Chem. Soc.*, **86**, 3735 (1964); M. Friedman, J. F. Cavins, and J. S. Wall, *J. Am. Chem. Soc.*, **87**, 3672 (1965); *J. Am. Chem. Soc.*, **89**, 4709 (1967).
51. J. F. Cavins and M. Friedman, *J. Biol. Chem.*, **243**, 3357 (1968).
52. H. Esterbauer, E. Schauenstein, and H. Zollner, Symposium, Austrian Biochemical Society, Nov. 1974; H. Zollner, H. Esterbauer, and E. Schauenstein, *Z. Krebsforsch*, **83**, 27 (1975).
53. K. Kollaritsch, Thesis, University of Graz, 1973.
54. S. Seeber, P. Warnecke, and U. Weser, *Z. Krebsforsch.*, **72**, 137 (1969).
55. E. Boyland and E. Chasseau, *Biochem. J.*, **109**, 651 (1968).
56. J. Frazer, M. Peters, and M. Hardinge, *Molec. Pharmacol.*, **3**, 247 (1967).

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