## On the Stability of N-Ethylmaleimide Alkylated Sulfhydrylgroups

N-ethylmaleimide (NEM) is widely used for the alkylation of free and protein-bound sulfhydryl groups (SH). This addition of NEM occurs instantaneously and has so far been assumed to be irreversible. Recently, however, BEUTLER et al.[1] presented evidence that the glutathione-NEM complex (GSH-NEM), formed in red cells, dissociates slowly at pH 7.4 and more rapidly at pH 8.2. The released products showed properties different from NEM and were postulated, by the authors, to be either hydroxy-N-ethylsuccinimide and/or N-ethylsuccinimide. In the following we wish to report observations suggesting other possible degradation of SH-NEM complexes at pH values above neutrality.

When H-meromyosin was labelled for 10 min at pH 7.4 with $^{14}$C-NEM (Schwartz Bioresearch, N.Y.) in the presence of 2 *M* guanidine-HCl, hydrolyzed with trypsin for 24 h in 0.1 *M* ammoniumcarbonate buffer, pH 8.5, and the resulting peptides were separated in a two-dimensional system[2], the radioautograms of the labelled peptides consistently gave the patterns shown in Figure 1a. When, however, the tryptic digestion preceded the labelling with $^{14}$C-NEM at pH 7.4, a different (but in itself reproducible) pattern was obtained (Figure 1b). Although the number of peptides remained the same, the peptides of the latter experiment migrated considerably faster in the second (chromatographic) dimension.

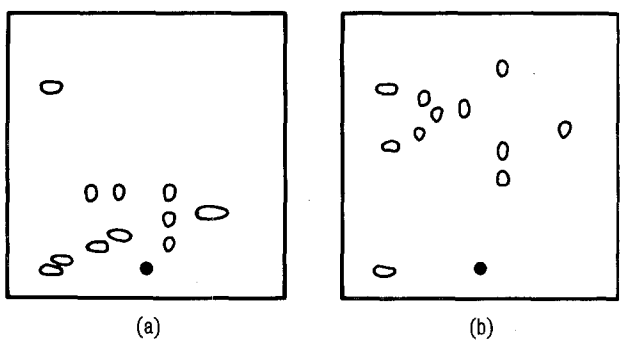


Fig. 1. Tracing of a radioautogram of $^{14}$C-NEM labelled H-meromyosin after tryptic hydrolysis and two-dimensional separation[2]. a) Labelling before tryptic hydrolysis, b) labelling after tryptic hydrolysis.

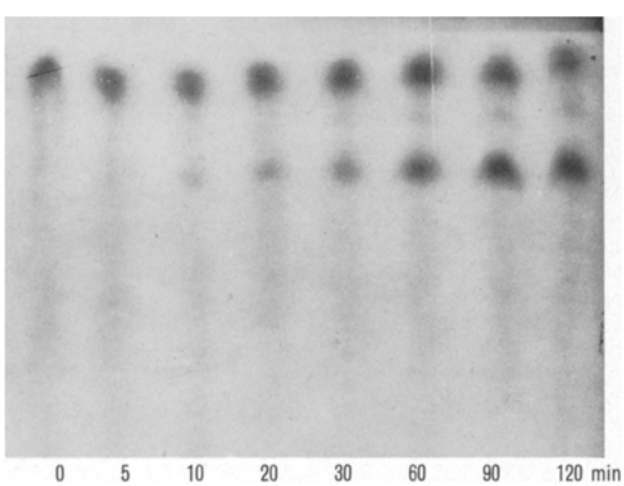


Fig. 2. Radioautogram of $^{14}$C-NEM after incubation in 0.1 *M* ammonium carbonate buffer, pH 8.5, from 0–120 min. Chromatography on Silicagel S, solvent system see[2].

After it had been established (both by measuring the uptake of NaOH in an autotitrator and the release of non-trichloroacetic acid precipitable nitrogen) that the proteinase activity per se was not influenced by the presence or absence of NEM, we concluded that the protein-bound NEM underwent chemical modification under the conditions of tryptic hydrolysis.

To test this concept, we first incubated the $^{14}$C-NEM used in this experiment with 0.1 *M* ammonium carbonate buffer, pH 8.5. Irrespective of the presence or absence of the proteolytic enzyme, on thin-layer plates the gradual appearance of a second radioactive spot was noted when aliquots of the mixture were separated after 20–120 min of incubation (Figure 2). Concurrent with the appearance of this second substance, a decrease of the NEM-specific $E_{max}$ at 303 nm was observed (Figure 3), which is presumably due to hydrolysis of the imide linkage at pH values above neutrality[3]. Similarly, the $E_{max}$ of the GSH-NEM complex at 258 nm disappeared after incubation at pH 8.5, although at a much slower rate (Figure 3). When aliquots of this incubation mixture were spotted at various intervals on thinlayer plates and developed after 24 h, only 1 ninhydrin-positive spot (d) was seen at 0 time, and as this was quite different from GSH (c) and its oxidized form GSSG (a), it was attributed to the GSH-NEM complex. In samples spotted at 2–6 h, the gradual appearance of spot (b) was noted, whereas spot (d) could not be traced after 24 h. The other ninhydrin-positive spot (e) noted between 2–24 h was later identified as N-ethylmaleamic acid (NEMA), an hydrolysis product of the ninhydrin-negative NEM, which had been used in a slight molar excess in these experiments (Figure 4).

These data suggest that free and peptide- or protein-bound NEM hydrolyzes with varying rates at pH values above neutrality, and that this process is independent of the presence of proteolytic enzymes. Since unsubstituted succinimide has an $E_{max}$ at 240 nm, the disappearance of the $E_{max}$ at 258 nm may well represent a hydrolysis of the substituted succinimide ring, the splitting of which seems

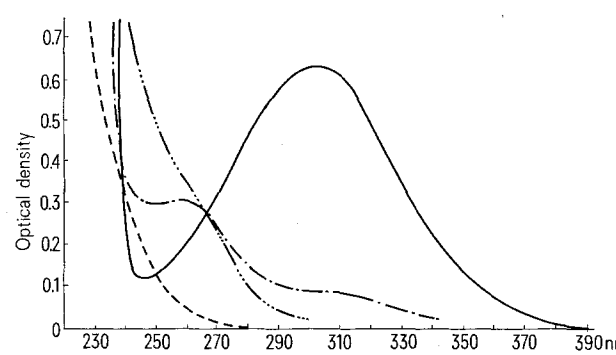


Fig. 3. Absorption spectra of NEM (—), GSH (---), GSH-NEM at pH 6.7 (-.-.-) and GSH-NEM after 24 h at pH 8.5 (·····). Concentration: 1 $\mu M$/ml.

[1] E. BEUTLER, S. K. SRIVASTAVA and C. WEST, Biochem. biophys. Res. Commun. *38*, 341 (1970).
[2] U. GRÖSCHEL-STEWART und F. TURBA, Biochem. Z. *337*, 104 (1963).
[3] J. D. GREGORY, J. Am. chem. Soc. *77*, 3922 (1955).

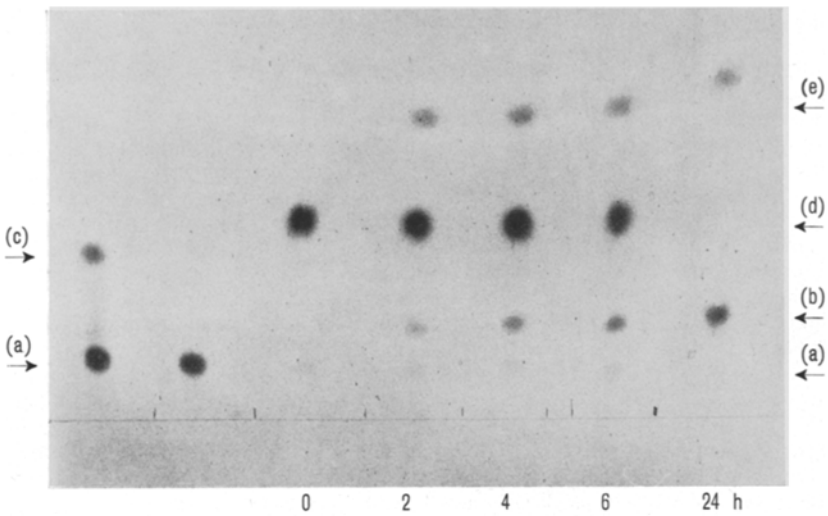


Fig. 4. Chromatographic separation of the GSH-NEM complex incubated at pH 8.5 from 0–24 h. (a), GSSG; (b), hydrolysis product of GSH-NEM; (c), GSH; (d), GSH-NEM; (e), hydrolysis product of excessive NEM (NEMA). Stained with ninhydrin.

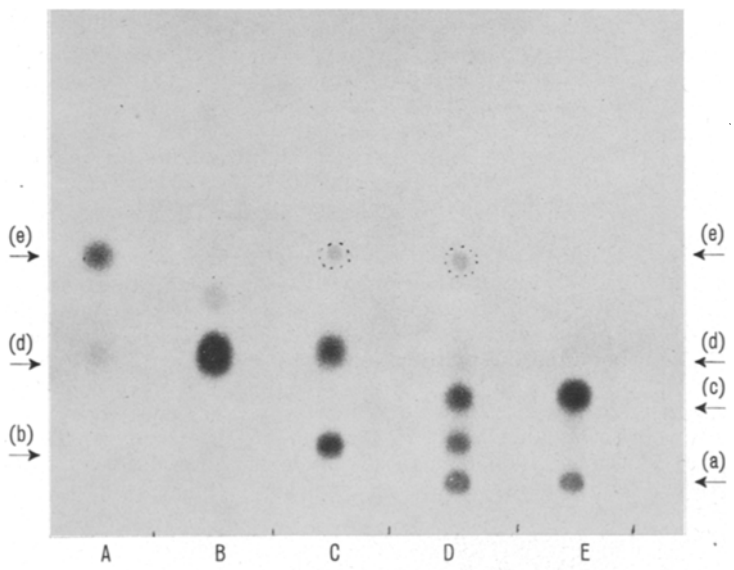


Fig. 5. Thin-layer chromatogram of A, NEMA (e); B, GSH-NEM (d); C, GSH-NEM hydrolyzed at pH 8.5 (b, d, e); D, reaction products of GSH and NEMA (a, b, c, e); E, GSH (c) and GSSG (a). Stained with ninhydrin.

to be complete after 24 h, as judged from the total conversion of GSH-NEM (d) into (b) with its lower Rf-value and as also seen in the differernt patterns obtained in Figure 1, a) and b).

In an attempt better to identify these hydrolysis products, N-ethylmaleamic acid was synthesized[4]. In thin-layer chromatography, this substance had an Rf-value identical with spot (e) obtained after alkaline hydrolysis of NEM (Figure 5; A, C and D). Since NEMA will slowly combine with GSH[3], we incubated the 2 substances for 72 h at pH 7.4 and identified the following reaction products on the chromatogram (Figure 5, D): GSH (c), GSSG (a), NEMA (e), the reaction product of GSH-NEMA (b) with a Rf-value identical to that of hydrolyzed GSH-NEM (C, (b)). As was expected, GSH-NEM itself (B, (d)) was not present.

In contrast to BEUTLER et al.[1], we never obtained evidence of a dissociation of the SH-NEM complex. GSH or GSSG do not reappear in our chromatographic separations, and we cannot conclude from our spectrophotometric data that intact succinimide rings were released. Although BEUTLER could show that this release occurs non-enzymatically, it cannot be excluded that the degradative mechanisms in the in vivo red cell system is entirely different from the ones involved in our in vitro incubation mixtures.

From our data we conclude that exposure of SH-bound NEM to pH values above neutrality (e.g. tryptic hydrolysis) will cause cleavage of the imide ring, as is in accord with the results found with free NEM[3]. This will result in an alteration in the chromatographic behaviour of the peptides to be investigated. Furthermore, it must be kept in mind that labelling of biologically active proteins with NEM and the resulting loss of their biological activity (e.g.[5,6]) may be due not solely to specific SH-blockade, but

[4] A. PIUTTI and E. GIUSTINIANI, Gaz. chim. ital. *26*, 438 (1896).
[5] U. GRÖSCHEL-STEWART, H. RÜDIGER und F. TURBA, Biochem. Z. *339*, 539 (1964).
[6] U. GRÖSCHEL-STEWART, Experientia *25*, 601 (1969).

also to an increase of carboxyl-residues close to or at the protein's active site.

*Zusammenfassung.* Anhand von Modellversuchen konnte gezeigt werden, dass SH-gebundenes N-Äthylmaleinimid bei pH-Werten über dem Neutralpunkt allmählich zur entsprechenden N-Äthylsuccinamidsäure hydrolisiert. Dadurch verändert sich das chromatographische Verhalten NÄM-markierter Peptide und möglicherweise auch die biologische Aktivität NÄM-blockierter Proteine.

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## The Amine and Amino Acid Composition in the Retzius Cells of the Leech *Hirudo medicinalis*

It has become clear in recent years that precise biochemical information on the functioning of nervous systems is best obtained from experiments on individual neurons. The so-called 'collossal' or Retzius cells[1] in each abdominal ganglion of the leech *Hirudo medicinalis* are particularly suitable for biochemical studies because they can be dissected from their surrounding tissues.

These neurons are globe-shaped with a diameter of up to 80 µm and are known to contain serotonin[2,3]. Although the Retzius cells can vary in size, their constant position and superficial location allow them to be easily distinguished from the surrounding cells in living preparations. We applied the microbiochemical technique developed by Neuhoff et al.[4,5] to investigate the amine and amino acid content of the Retzius cells and also the abdominal nerve chain of the leech. The content of the Retzius cells is compared with that of the serotonin-containing neuron in the metacerebral ganglion of the snail which has been described elsewhere[6,7].

*Materials and method.* Active leeches were obtained from a local dealer and groups of about 7 ganglia were isolated as described by Walker[8]. The ganglia were pinned, ventral side up onto the base of a small dissecting dish containing a sheet of plastic, filled with cold saline[9], and were placed on a specially cooled dissecting microscope stage. Single Retzius cells (there are two in each abdominal ganglion of the nerve chain) were carefully freed from the surrounding nerve tissues using thin tungsten needles and sharpened forceps. Cells were then individually lifted free by suction from the ultra thin tip (15 µm in diameter) of a glass pipette, attached to the mouth by rubber tubing. 40 neurons were transferred to a 10 µl capillary (Drummond microcap) which had been heat-sealed at one end and contained 3 µl of 0.05 *M* sodium bicarbonate pH 10. The cells were then homogenized using a special nerve canal drill, 3 µl acetone were added to precipitate the proteins and the capillary was then centrifuged[6]. The supernatant was next transferred to a clean microglass tube and reacted with an equal volume of 5 µ*M*/ml $^{14}$C-dansyl-chloride (Schwarz/Mann, Orangeburg, New York) specific activity 49 mC/m*M*). A number of trial experiments were initially carried out to determine the concentration of $^{14}$C-dansyl-chloride required, since it is known that this is of importance in order to obtain optimal reaction conditions[10]. After incubating the micro-glass tube and its contents for 30 min at 37 °C, the mixture was evaporated to dryness under reduced pressure and the dansylated substances resuspended in 2 µl acetone/acetic acid (3:2 v/v). Aliquots of 0.5 µl were spotted onto a corner of a 3 × 3 cm polyamide layer and developed by two dimensional chromatography (Figures 1a and 2a). A third chromatography in the second dimension was required to separate alanine from dansyl-$NH_2$, aspartic acid from glutamic acid and serine from threonine, glutamine and asparagine (Figures 1b and 2b), although this resulted in a poor definition of some other substances. The chromatographic procedure, a method for obtaining autoradiograms and for the quantitative measurement of each substance on a single microchromatogram is described elsewhere[4–6]. In addition, after the Retzius cells had been dissected, the abdominal nerve chain was homogenized (1 mg tissue per 10 µl sodium bicarbonate) and centrifuged and 3 µl of the supernatant was then reacted with the same quantity of 5 µ*M*/ml $^{14}$C-dansyl-chloride and incubated for 30 min at 37 °C. The mixture was then evaporated to dryness, redissolved in 5 µl acetone/acetic acid and applied in aliquots of 0.5 µl to microchromatograms for analysis.

*Results and discussion.* Autoradiograms showing the occurrence of substances which react with dansyl chloride are shown in Figures 1 and 2. Each substance can be identified by comparing the spot numbers on the maps with those shown in the Table. The radioactivity associated with each substance from chromatograms of 2 different experiments for both Retzius cells and abdominal nerve chain are also shown in the Table. Comparison of the percentage composition of each dansyl derivative in the different experiments demonstrates that reproducible results are obtained. Since the method depends on the reaction of dansyl-chloride with aliphatic amino or hydroxy groups to form fluorescing compounds at alkaline pH, a single substance which contains an aliphatic amino and hydroxy group, as does serotonin, can provide 3 possible fluorescing compounds, i.e. N-, OH- and *Bis*-form. In practice, however, the number of compounds produced depends on the experimental conditions[4,11]. One of the most striking features of these

[1] G. Retzius, Biol. Unters. *2*, 13 (1891).
[2] G. A. Kerkut, C. B. Sedden and R. J. Walker, Comp. Biochem. Physiol. *21*, 687 (1969).
[3] S. Rude, R. E. Coggeshall and L. S. van Orden III, J. Cell Biol. *41*, 832 (1969).
[4] V. Neuhoff, in *Recent Advances in Quanittative Histo- and Cytochemistry* (Eds. U. C. Dubach and U. Schmidt, Hans Huber Publishers, Bern 1971), p. 109.
[5] N. N. Osborne, G. Briel and V. Neuhoff, Int. J. Neurosci. *1*, 265 (1971).
[6] G. Briel, V. Neuhoff and N. N. Osborne, Int. J. Neurosci. *2*, 129 (1971).
[7] N. N. Osborne, in Proc. IVth European Malacol. Congress, Geneva 1971, in press.
[8] R. J. Walker, in *Neurobiology in Invertebrates* (Ed. J. Salánki, Plenum Press, New York 1968), p. 227.
[9] S. Kuffler and D. Potter, J. Neurophysiol. *27*, 290 (1964).
[10] G. Briel and V. Neuhoff, sent to the Editors.
[11] G. Briel, Doctoral Thesis, University Göttingen 1972.