THE APPLICATION OF SIMPLE PAPER CHROMATOGRAPHY
TO THE STUDY OF GLUTATHIONE
IN THE RAT EPIDERMIS

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The use of paper chromatography has been demonstrated to be a simple
and efficient means for the detection and separation of glutathione from
the constituent amino acids and the related metabolite. The use of N-
ethylmaleimide to stabilize the sulfhydryl group during chromatography
was found to be a marked advance. This technic was applied to the study
of glutathione in the epidermis. The incorporation of 14C-glycine into
 glutathione was demonstrated under the homogenate of rat epidermis.

Glutathione (GSH) is known to widely
distribute among living cell. GSH has
been regarded to play an important role
in cellular hydrogen and electron trans-
port, because of the case of interconver-
sion of sulphydryl and disulfide form
of the compound. Since the presence of
water-soluble sulphydryl compound in
the epidermis was first described by
Rothman 1, the physiological significa-
cance of GSH has been recognized in
the field of skin biochemistry. The
synthesis of GSH in human erythrocyte
was demonstrated by using labeled
constituent amino acids of GSH 2, 3, 4, but GSH in the epidermis has not been
focused. Gutcho and Lauf er 5 summarized the progress on the selection of
suitable solvents for chromatographic
separation of GSH. The present experi-
ment was attempted to adapt the paper
chromatographic technic for the study
of GSH in the epidermis.

MATERIAL AND METHODS

1. Paper chromatography

The unequivocal separation of GSH
from the constituent amino acids and
the related metabolite was carried out
by paper chromatography described
with Gutcho and Lauf er 5. The use of
N-ethyl-maleimide (NEM) to stabilize
the sulphydryl (SH) group during chro-
matography was a marked advance. To
stabilize the SH group prior to chro-
mography, 50 μl of sample was reacted
with same volume of 5 × 10⁻²M NEM.
Paper chromatographic separation was
carried out on TOYO No.7 filter paper.
The sheet of 18 by 5.5 cm was divided
into four lanes. The sample to be chro-
matographed was deposited on a starting
line at 5 cm from the bottom of the
sheet, with a micropipet of 1 or 2 μl
capacity. For the identification of the separated substance, authentic GSH treated with NEM (NEM-GSH) was also chromatographed as a reference. The paper was stapled to form a cylinder with edge (not quite touching), which was then placed upright in a glass cylinder, the bottom of which contains 20 ml of developing solvent. The developing solvent used in the present experiment consisted of tertiary butanol (TB), formic acid (F), and distilled water (W) (70:15:15 vol/vol). The top of cylinder was sealed with glass-plate, and the solvent permitted to ascend the paper sheet at room temperature. The sheet was removed when the front of solvent was reached almost to the top of the paper, and it was then hung to dry. Separation for 18 hours at 25°C gave generally satisfactory resolution. For the location of the separated substances, the purple colored spot was developed as a routine chromatography by 0.25 % ninhydrine-acetone solution. About 2 μg of GSH could be detected.

2. Epidermal material

Albino rats were killed by cutting the carotid arteries, and epidermal materials were prepared from rats’ back skin. The hair from the animal’s back was manually plucked, and the skin was excised. The subcutaneous fat and connective tissue were scraped off with a scalpel, and the epidermis was separated from the dermis by Van Scott's mechanical strech technic. The separated epidermis was immediately homogenized with 0.1 M phosphate buffer pH 6.9 (5 ml per 1 g wet weight) until it formed a fine emulsion. The homogenate was centrifuged at 1,000×g for 15 minutes to separate the cellular debris. The resulting supernatant is hereafter referred to as the epidermal supernatant.

3. Incorporation of 14C-glycine into GSH

The epidermal supernatant was incubated with 14C-glycine (20 μc) at 37°C in 0.1 M phosphate buffer (pH 6.9). Ten per cent of trichloroacetic acid solution was added to the incubation mixture to make a final concentration of 4 %, at the desired period of incubation time: 1/2, 1, 3, 4 and 5 hours incubation. After standing for 30 minutes in the ice-cold water, the precipitate was removed by centrifugation at 2,700×g for 15 minutes, and the acid-soluble supernatant was separated. The resulting supernatant was used as the sample to be chromatographed. Fifty μl of supernatant treated with NEM was subjected for the paper chromatography. After paper chromatography, the reference lane for authentic NEM-GSH was cut off the sheet of paper, the purple colored spot was developed with ninhydrine and the location of authentic NEM-GSH was detected. The corresponding area to the authentic NEM-GSH was cut off from each lane to use for the following radioactive assay.

The corresponding area of each chromatographic lane was put into the vial-glass, and the radioactivity level was determined with filter paper lysing under 5 ml of dioxan scintillator. The dioxan scintillator consisted of 2 - 5 - diphenyloxazole (1.3 g), 1 - 4 - bis - 2 - (5 - phenyloxazoyl) benzene (0.026 g), naphthalene (20.5 g), ethanol (60 ml), toluene (100 ml) and dioxane (100 ml).

4. Determination of Protein

The protein content in the epidermal supernatant was determined by Lowry's method stanarized with bovine serum albumin. The count of radioactivity was expressed per mg protein.
RESULTS AND DISCUSSION

1. Separation of the reduced and oxidized glutathione

GSH is easily oxidized to GSSG. The application of GSH to the chromatography showed a trace spot of GSSG, because of the tendency of GSH to become GSSG on the chromatographic procedure.

In 1964 Hochberg et al. reported the electrophoretic separation of GSH, GSSG on the study of GSH metabolism of human erythrocyte. GSH without stabilization with NEM was separated from GSSG, glycine, glutamic acid, aspartic acid and lysine on the electrophoresis in 0.02 M phosphate buffer (pH 6.9).

But, in the homogenized rat epidermis, GSH and the related metabolite were not well separated as shown in human red cell.

Nine solvent systems have been reported by Gutcho and Lauffer as most effective for use in separating and detecting the GSH, GSSG and their hydrolysis products. The TBFW solvent system was selected to provide adequate separation for our present experiment. The spots for GSH and GSSG on the TBFW solvent system were identified respectively by their paper chromatographic mobilities. GSSG remained almost at the starting point.

States and Segal described that cysteine and GSH were combined with NEM to form the stable adducts. The thin-layer chromatography was applied to study these adducts in metabolic system.

In our present experiment, GSH was also previously stabilized with NEM for blocking the SH group of GSH. By using the pretreatment with NEM, the spot for GSSG was not appeared on the chromatogram as the result of autoxidation of GSH.

<table>
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<tr>
<th>Rf value</th>
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<tr>
<td>Glutathione (GSH)</td>
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<tr>
<td>oxidized glutathione (GSSG)</td>
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<tr>
<td>Glutathione pretreated with NEM</td>
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2. Separation of GSH from the related metabolites

As a glycine is altered metabolically to other compounds such as serine, cysteine/ cystine and nucleic acid. The study of GSH biosynthesis by using glycine is required to separate from other related compounds altered metabolically from glycine. The initial experiment was attempted to demonstrate if GSH could be separated from the related compounds. Figure 1 shows the chromatogram of mixture of glycine, serine, glutamic acid, cystine and cysteine. The TBFW solvent system shows poor separation of GSH and the related amino acids. However, by using the NEM combination, the NEM-GSH and the related amino acids are separable.

![Fig. 1 Rf values (TBFW solvent)]
The use of paper chromatography has proved to be a simple and efficient means for the detection and separation of GSH, constituent amino acids and the related metabolites. The thin-layer chromatographic separation for metabolic and enzymatic studies of glutathione in the epidermis is being investigated, by using the NEM-GSH.

3. Incorporation of $^{14}$C-glycine into glutathione

The incorporation of $^{14}$C-glycine into GSH was carried out by freshly prepared homogenate of rat epidermis. The GSH synthetized was separated and identified by paper chromatographic technic described above. The time course of the incorporation $^{14}$C-glycine into GSH was shown in Fig. 2. The incorporation into GSH was relatively slow by 3 hours incubation, but was stimulated remarkably at the 5th hour after incubation. From these experimental results, the enzyme which catalyze the formation of GSH was found in the epidermis as well as of the other organs in mammalian.

Glutathione and $^{14}$C-glycine were supplied from the Yamanouchi Pharmaceutical Company Ltd. Tokyo.

REFERENCES


