Non-enzymatic Reduction of Tetrazolium Salts by Sulfhydryl Groups.

Using liver, kidney, adrenal and skeletal muscle of the rat, ZIMMERMAN and PEARSE (1959) reported that tissue-SH, either protein-bound or present in small molecules like glutathione, is able to reduce exogenous diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) at alkaline pH levels and that the resulting reduced DPN (DPNH) or reduced TPN (TPNH) can reduce tetrazolium salts through the corresponding diaphorases. They called this reaction 'nothing dehydrogenase' (BACKER 1955).

During the course of histochemical studies on the cytochrome-linked and coenzyme-linked dehydrogenases in the skin (HASHIMOTO and OGAWA 1960), we also encountered a substrate-independent pH-dependent reduction of tetrazolium salts in the actively growing hair follicles of the young rat. The strong tetrazolium-reducing zone in the hair follicles in the anagen stage of the hair cycle begins above the hair bulb and fades out below the level of the tip of the sebaceous gland, and this zone corresponds to the parakeratotic zone of the hair root or 'keratogenous zone' of GIROUD and BULLIARD (1930). The keratogenous zone of the hair root and the subcorneal thin layer (mostly coinciding with the granular layer) are known to be the sites of keratinization, namely the transformation of SH groups to S-S groups (cysteine to cystine) to form disulfide cross-linkages between micells (CHEVREMONT and FREDERIC 1943, GIROUD and LEBLOND 1951, HARDY 1952). There seems to be little doubt that protein-bound SH groups are involved in this non-enzymatic reduction of tetrazolium salts in these zones of the skin. ROGERS (1953), and FORGISANO and MONTAGNA (1954) demonstrated SH groups in these zones using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) which has higher half wave potential than nitrotoetrazolium chloride (nitro-NT). With nitro-NT used in the present investigation SH groups could only be demonstrated in the keratogenous zone of the hair.

With regard to the hydrogen pathways from SH groups to tetrazolium salts, however, there appears to be a direct transfer of hydrogen from SH to tetrazolium salts in addition to the pathway mediated through DPN or TPN diaphorases as described by ZIMMERMAN and PEARSE (1959), since the preliminary work revealed the positive reaction in the keratogenous zone following incubation of tissue sections with a medium containing tetrazolium salts only at alkaline pH.

Our present observation is plotted to elucidate the mechanism of this non-enzym-
matic reduction of tetrazolium salts by SH groups in the rat hair in terms of the hydrogen pathways from SH to tetrazolium salts.

I. Materials and Methods.

Animal materials.

One new-born, one five-day-old, one ten-day-old, one twelve-day-old and two twenty-day-old rats of Wistar strain were used in the present investigation. Rats were killed by a blow on the neck without anesthesia. The skin was stripped of hair and the specimens were taken from the back. Skin was removed from the backs of new-born, five-day-old, and ten-day-old rats while they were still alive. Skin specimens were cut into small square pieces, immersed briefly in ice-cold SÖRENSEN’s 0.1 M phosphate buffer, pH 7.6 (for the study of succinic dehydrogenase system and nothing dehydrogenase) and pH 7.4 (for coenzyme-linked dehydrogenases), and sectioned in approximately 20 μ thick slices by a freezing microtome. In some cases the skin specimens were fixed in cold acetone (2°C, 10 minutes) prior to sectioning with a freezing microtome.

Staining methods.

The succinic dehydrogenase system was histochemically demonstrated by OGAWA and ZIMMERMAN’s method (OGAWA and ZIMMERMAN 1959), and NACHLAS, WALKER and SELIGMAN’s method was applied to demonstrate coenzyme-linked dehydrogenases such as lactic and malic dehydrogenase systems (NACHLAS, WALKER and SELIGMAN 1958).

For the study of non-enzymatic reduction of tetrazolium salts (‘nothing dehydrogenase’), the following substances were employed. Buffers: 0.1 M SÖRENSEN’s phosphate buffer, SÖRENSEN’s borate buffer; Tetrazolium salt: nitroneotetrazolium chloride (nitro-NT); Coenzyme: DPN; Sulfhydryl inhibitor: iodoacetic acid; Cytochrome inhibitors: sodium cyanide, sodium azide.

Using these substances, the following incubating media were prepared.

Medium 1 (nitro NT only):

- 0.1 M phosphate buffer, pH 7.6: 6 ml
- 0.1% nitro-NT: 2 ml
- Distilled water: 2 ml

Medium 2 (nitro-NT, SH inhibitor):

- Iodoacetic acid (0.01, 0.05 and 0.1 M in the final concentration of the incubating medium) was added to medium 1.

Medium 3 (nitro-NT, cytochrome inhibitor):

- Sodium cyanide or sodium azide (0.01, 0.05, and 0.1 M in the final concentration) was added to medium 1.

Medium 4 (nitro-NT, SH inhibitor, DPN):

- DPN 2.5 mg was added to medium 1.

Medium 5 (nitro-NT, SH inhibitor, DPN):

- DPN 2.5 mg was added to medium 2.

Medium 6 (nitro-NT, cytochrome inhibitor, DPN):

- DPN 2.5 mg was added to medium 3.
Medium 7 (nitro-NT, SH inhibitor, cytochrome inhibitor, DPN):

Iodoacetate (0.01 M in the final concentration) was added to medium 6.

The frozen sections of skin were incubated in these media at 37°C for 3 hours, rinsed briefly in the phosphate buffer, fixed in 10% neutral formalin for 30 to 60 minutes and mounted in glycerine-jelly for microscopic observation.

The effect of pH on the non-enzymatic reduction of tetrazolium salts by SH groups was then investigated using medium 1. Sörensen's 0.1M phosphate buffer was used for the observation of the effect of pH 4.4 to 10.0 (pH adjusted with 2N NaOH, if necessary) and Sörensen's borate buffer was used for pH 10.0 to 13.0.

Reactivity of vitro-NT towards various S-containing substances in vitro.

In order to observe the direct reduction of tetrazolium salts by SH groups, various S-containing compounds (cysteine, cystine, methionine, reduced and oxidized glutathiones, sodium hydrosulfide, ethyl mercaptan, 2-mercaptobenzothiazole, 2-mercaptobenzimidazole, thioglycerole and thioglycolic acid in the final concentration of 0.01 M) were added to the medium 1 in vitro. Incubation was carried out at 37°C for 3 hours as in the case of tissue sections and the color development (formation of tetrazolium formazan) was investigated. In some cases sodium cyanide (0.01 M in the final concentration) and/or iodoacetic acid (0.01 M in the final concentration) was added to the medium.

II. Results.

A. Distribution of the succinic, malic and lactic dehydrogenase systems in the rat skin.

The main distribution of the succinic dehydrogenase system in the adult rat skin was in the basal and squamous cell layers of the epidermis, occasionally up to the granular layer, peripheral (generative or indifferent) cells of acini of the sebaceous glands, inner and outer root sheaths, bulb and matrix of the hair, dermal muscles (both strongly positive red and less strongly positive white muscle fibers), and arrector pili. The central part of the acini of the sebaceous glands and subcutaneous fatty tissue were also stained pink. Pappillae were always unstained. The cortex and medulla of keratogenous zone of the hair produced intense dark-red staining which was easily distinguished from the granular enzymatic staining (Fig. 1).

The distribution of lactic and malic dehydrogenase systems was essentially the same as that of succinic dehydrogenase, but the staining reactions were much more intense, and the stainability of subcorneal thin layer was much more pronounced than in succinic dehydrogenase stain and the capillary elements and connective tissue cells, especially the mast cells in upper dermis, were also stained.

Sebaceous glands and subcutaneous fatty tissue were not stained any more following fixation of the specimens in cold acetone. The positive reaction observed in the keratogenous zone of the hair was completely abolished by a simultaneous addition of iodoacetate, a SH inhibitor, in the incubating medium (Fig. 2).
B. Non enzymatic reduction of tetrazolium salts (nothing dehydrogenase) in tissue sections.

An intense reaction in the keratogenous zone was observed in the skin specimens incubated in medium 1 (see Table 1). Specimens treated in medium 2, an inhibition...
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of reaction in the keratogenous zone, almost complete with the concentration of 0.01 M of iodoacetate (SH inhibitor) and complete by 0.05 M, was observed. Specimens incubated in the medium 3 were stained in the similar fashion as those incubated in the medium 1, when sodium azide was used as a cytochrome inhibitor. However, when sodium cyanide, 0.01 M in the final concentration, was used, the positive reaction in the keratogenous zone was more striking than when sodium azide was used. In addition the epidermis had a positive reaction. It was found that sodium cyanide higher than 0.01 M in the final concentration of the incubating medium showed its own reducing power on tetrazolium salts, resulting in the formation of tetrazolium formazan in the medium. The addition of DPN alone (medium 4) intensified the staining reaction of the keratogenous zone, but the simultaneous addition of 0.01 M iodoacetate (medium 5) annihilated this effect and, furthermore, the keratogenous zone was not stained at all.

Addition of a cytochrome inhibitor, sodium azide, to the medium containing nitro-NT and DPN did not cause any specific change in the staining reaction of the keratogenous zone. Specimens incubated in medium 7 showed no positive reaction either in the keratogenous zone or in the epidermis.

The intensity of the reaction in the keratogenous zone depended on the pH of the incubation medium. Below pH 4.4 no reduction of tetrazolium salts occurred. At pH 4.4 the reaction became very weakly positive (Fig. 3). Around at pH 7.0 the reaction was considerably increased and it was rapidly intensified in the alkaline side of neutrality as pH rose up to pH 13, which was the highest pH tested and produced purplish red color in the medium when a small piece of tissue was added (Figs. 4, 5).

C. Reactivity of nitro-NT towards various S-containing substances in vitro.

The results are presented in Table 2. It can be observed that L-cysteine and thioglycerol reduced nitro-NT immediately following the mixture of the substances,

Table 1. Effects of various compounds on the non-enzymatic reduction of nitro-NT in the skin of 12-day-old rat.

<table>
<thead>
<tr>
<th>Tissue sections incubated with*</th>
<th>Nitro-NT</th>
<th>Nitro-NT, SH inhibitor</th>
<th>Nitro-NT, Cytochrome inhibitor</th>
<th>Nitro-NT, DPN</th>
<th>Nitro-NT, Cytochrome inhibitor (NaN₃)</th>
<th>Nitro-NT, DPN, Cytochrome inhibitor, SH inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratogenous zone</td>
<td>++</td>
<td>0</td>
<td>NaCN</td>
<td>+++</td>
<td>0</td>
<td>+++</td>
</tr>
<tr>
<td>Epidermis</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The composition of the incubation medium is described in the text.
Symbols: 0 no reaction (negative), + weak reaction, ++ moderate reaction, +++ strong reaction.

The results are presented in Table 2. It can be observed that L-cysteine and thioglycerol reduced nitro-NT immediately following the mixture of the substances,
Fig. 3. Stained in 0.1% nitronetetrazolium chloride (nitro-NT) solution adjusted to pH 4.4. Very weakly stained keratogenous zone of the hair is demonstrated. There is no remarkable difference of staining reaction between the keratogenous zone and lower follicles. 20-day-old rat skin. ×150.

Fig. 4. Stained in 0.1% nitro-NT solution adjusted to pH 8.0. Much intensified staining reaction of the keratogenous zone. 20-day-old rat skin. ×150.

Fig. 5. Stained in 0.1% nitro-NT solution adjusted to pH 13.0. Further intensified reaction of the keratogenous zone than in Fig. 4. 20-day-old rat skin. ×200.
that ethyl mercaptan reduced it 15 to 20 minutes after mixture, and that the reduced glutathione reduced it approximately 5 hours following mixture. The reducing power of these substances was completely blocked by the simultaneous addition of iodoacetate (SH inhibitor) to the medium. L-cystine, DL-methionine, sodium hydrosulfide, thioglycolic acid, 2-mercaptobenzothiazole, 2-mercaptopenimidazole, which revealed negative reaction in the mixture with nito-NT alone, reduced nitro-NT by the concomitant addition of sodium cyanide, 0.1 M in the final concentration, to the medium. Oxidized glutathione did not have any reducing potentiality at all.

III. Discussion.

The distribution and activities of succinic, malic and lactic dehydrogenase systems are discussed in detail elsewhere (HASHIMOTO and OGAWA 1960).

The reduction of nitro-NT observed in the keratogenous zone of the rat skin is by no means due to enzymatic activity, since the reaction remained positive following

| Table 2. Reactivity of nitro-NT towards S-containing substances in vitro. |
|---|---|---|
| L-Cysteine | HSCH₂CH (NH₂) COOH | ++ ++ (immediately) | Nitro-NT | Nitro-NT, Sodium cyanide | Nitro-NT, SH inhibitor |
| L-Cystine | (COOHCH (NH₂) CH₂S)₂ | 0 | ++ (over night) | --- | --- |
| Ethyl mercaptan | C₂H₅SH | ++ (15-20 min.) | --- | 0 |
| Glutathione (reduced) | HOOC (CH₂)₂ CHNH₂CO-NHCH(CH₂SH/CONHCH₂ COOH (G-SH)) | ++ (5 hrs.) | --- | 0 |
| Glutathione (oxidized) | G-S-S-G | 0 | 0 | --- |
| DL-Methionine | CH₃S(CH₂)₂CH(NH₂)-CO-OH | 0 | ++ (over night) | --- |
| 2-Mercaptobenzothiazole | C₆H₅SCSHN | 0 | + (30-60 min.) | --- |
| 2-Mercaptopenimidazole | C₆H₅NHC (SH)N | 0 | + (30-60 min.) | --- |
| Sodium hydrosulfide | NaHS | 0 | +++ (immediately) | --- |
| Thioglycerol | HOCH₂CH (OH) CH₂SH | ++ (immediately) | --- | 0 |
| Thioglycolic acid | HSCH₂COOH | 0 | ++ (5 hrs.) | --- |

* The composition of the incubation medium is described in the text.
* Symbols as in Table 1.
incubation of tissue sections with the substrate-free incubating medium (see Table 1). The faint red stain seen in the sebaceous glands and the subcutaneous fatty tissue is also due not to enzymatic activity, but to the dissolving of formed formazans in the lipids (GODDARD and SELIGMAN 1953). This is evidenced by the fact that the staining was completely abolished after fixation of specimens in cold acetone.

PEARSE reported an alkaline tetrazolium reaction at pH 12.8 and 60°C using formalin-fixed tissue and classified non-enzymatic reduction of tetrazolium salts in the tissue into the following three categories: 1. sulfur-containing, 2. lipid- or lipofuscin-containing, 3. reducing sugar-containing (PEARSE 1954). Cysteine was indicated by him to be the main sulfur-containing compound in the tissue. However, it is most likely that cysteine is responsible for the direct non-enzymatic reduction of tetrazolium salts particularly in the keratogenous zone of the skin, since it has been known that SH groups are rich in the keratogenous zone (EISEN, MONTAGNA and CHASE 1953, ROGERS 1953, FORMISANO and MONTAGNA 1954, BARRNETT 1953), and that the keratinization is considered to be based on the chemical transformation of cysteine (SH) to cystine (S-S) (CHEVREMONT and FREDERIC 1943, GIROUD and LEBLOND 1951, HARDY 1952). Furthermore, as shown in Tables 1 and 2, it was observed in the present observation that the positive reaction in the keratogenous zone was blocked by addition of iodoacetate (SH inhibitor), that cysteine reduced nitro-NT in vitro, but cystine did not, until sodium cyanide was added to the mixture to convert cystine to cysteine, and that the positive formazan formation observed in the mixture of cysteine and nitro-NT was blocked by the simultaneous addition of iodoacetate even in vitro. These serial findings are favorable for the concept that cysteine rather than cystine is responsible for the non-enzymatic reduction of nitro-NT in the tissue. In addition to cysteine, reduced glutathione, ethyl mercaptan and thioglycerol, which also reduce nitro-NT in vitro, may be involved somehow in the non-enzymatic reduction of tetrazolium salts in the skin, though the detailed metabolic roles of these compounds are still unknown. The fact that the concomitant addition of DPN to nitro-NT (see Table 1) intensified the positive reaction in the keratogenous zone supports the idea of ZIMMERNAN and PEARSE (ZIMMERMAN and PEARSE 1959) that the hydrogens of SH groups are transferred to tetrazolium salts via DPN (or TPN) diaphorases. However, taking into consideration the evidence obtained in the present investigation that tetrazolium salts can be reduced by SH groups without DPN and this non-enzymatic reduction of tetrazolium salts can be blocked completely by iodoacetate both in tissue sections and in vitro (see Tables 1 and 2), there seems to be little doubt that there is a direct pathway of hydrogens of SH groups to tetrazolium salts in addition to the pathway via specific diaphorases. The redox potential (E°) of cysteine, being −0.14 (RACKER 1955), supports this concept. This concept of direct reduction of tetrazolium salts by SH groups has been in reality implicitly used in the histochemical demonstration of tissue-SH (ROGERS 1953). The cytochrome system appears to play an extremely minor, if any, role in the non-enzymatic reduction of tetrazolium salts in tissue, since the simultaneous addition of cytochrome inhibitor, particularly sodium azide, did not cause any change in the positive reaction observed in the keratogenous zone (see Table 1). The increased intensity seen with the addition of sodium cyanide, 0.01 M in the final concentration,
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seems to be due to the transformation of cystine (S-S) to cysteine (SH) converted by sodium cyanide (CHEVREMONT and FREDERIC 1943).

The main implication of the findings obtained in the present study is that there are at least two major pitfalls in the dehydrogenase histochemistry using tetrazolium salts: firstly, sodium cyanide higher than 0.01M in the final concentration of the incubation medium should not be used in the histochemical demonstration of the co-enzyme-linked dehydrogenases such as lactic or malic dehydrogenase systems, or else sodium cyanide reveals its own reducing activity on tetrazolium salts resulting in formazan formation in the medium which might give the false positive localization of enzymatic activity in tissues, and secondly, unless proper consideration or controls are taken for tissue-SH (nothing dehydrogenase), the precise localization of not only coenzyme-linked dehydrogenases but also cytochrome-linked dehydrogenases such as succinic dehydrogenase system may be difficult.

IV. Summary.

1. The non-enzymatic reduction of tetrazolium salts by SH groups was studied mainly in the keratogenous zone of the hair.
2. The effect of non-enzymatic reduction of tetrazolium salts by SH groups is pH-dependent, beginning at about pH 4.4, gradually increasing up to pH 7.0 and continuing to increase rapidly thereafter until pH 13 which is the highest alkaline pH tested. Addition of diphosphopyridine nucleotide (DPN) intensifies the reaction.
3. It was shown that several inorganic as well as organic substance having SH groups can reduce tetrazolium salts in vitro.
4. It is concluded that the hydrogen of SH groups can be transferred directly to tetrazolium salts in addition to the pathway via specific diaphorases as described by ZIMMERMAN and PEARSE (1959). The main implication of this fact is that the precise histochemical localization of not only coenzyme-linked dehydrogenases but also cytochrome-linked dehydrogenases may be difficult without proper controls for tissue-SH.
5. Since it was found that sodium cyanide higher than 0.01M in the final concentration of the incubation medium can also reduce tetrazolium salts, which may result in the false positive localization of enzymes, the concentration of the sodium cyanide should be kept lower than 0.01M in the incubation medium in the histochemical demonstration of coenzyme-linked dehydrogenases.

内容抄写。

Wistar 系ラッテの皮膚を用い、テトラゾリウム塩（ナイトロネオテトラゾリウム塩を用いた）の SH 基による非酵素的還元を、特に hydrogen transfer system の観点から追究した。皮膚内 SH（毛の角層に著明）のテトラゾリウム塩還元力は、浸漬液内にダイフォスフォビリジンニュクレオタイド（DPN）を加えると幾分増強するが、DPN を加えないことも反応が起こる事、サイトクロームオキシダーゼ抑制剤である窒化ナトリウム、またはシアノ化ナトリウムの影響を受けない事、及び、in vitro で SH を
有する諸物質が、直接（L-cysteine, ethyl mercaptan, reduced glutathione, thioglycerol）、或いはシアン化ナトリウムで活性化される事により（L-cystine, DL-methionine, 2-mercaptobenzothiazole, 2-mercaptobenzimidazole, sodium hydrosulfide, thioglycolic acid）、テトラゾリウム塩を還元する事等からして、SH 基の H は、DPN 或いは TPN ダイアフォレーズ系を介してテトラゾリウム塩に移行する道のみならず（ZIMMERMAN と PEARSE 1959）、直接、テトラゾリウム塩に移行する経路がある事が判った。

尚、SH 基の非酵素的還元反応は、浸漬溶液の pH によってその強さが左右される、即ち、pH 4.4 附近で始めて現われ、その後、pH 7.0 近辺は徐々に強まるが、アルカリ性溶液では還元力が急速に強まり、本研究で試みた最高 pH 値、pH 13.0 で最も強かった。

附加的所見として得られた事は、シアン化ナトリウムの浸漬液内最終濃度が、0.01M 以上の場合には、シアン化ナトリウム自身が、テトラゾリウム塩を還元する能力が有る事であり、この事実は、乳酸脱水素酵素系、リンゴ酸脱水素酵素系等の組織化学的検出の際用いるシアン化ナトリウムの量に注意を要する事を示唆している。

更に、テトラゾリウム塩を用い、諸脱水素酵素系の活性を検出する際、組織内 SH 基の存在を考慮に入れなければ、本来の酵素反応と、SH 基による非酵素的還元反応を混同し、酵素活性の判定を誤る恐れがある。皮膚の如く、SH 基の多い組織では特に慎重を期さなければならない。

References.