K. and Ogawa, K. : Arch. hist. jap., 21 : 239, 1961. 17) Zimmerman, H. and Pearse, A. G. E. : J. Histochem. Cytochem., 7 : 271, 1959.

Explanation of plates

- Fig. 1 : Normal human lens stained deep blue by DDD method of Barnett and Seligman.
 Fig. 2 : Advanced brown cataractous lens (DDD method). Sclerotic fibrous structure was stained deep brown showing a completely negative -SH group reaction, although the epithelium and the germinative zone remained positive.
 Fig. 3 : Advanced brown cataractous lens (RSR method of Pearse).
 Fig. 4 : Congenital cataractous lens (DDD method).

Discussion

Dr. K. Chin :

- 1) Were there any differences in reactions for SH group of protein of the lenticular epithelium between the cases treated with α -chymotrypsin at total extirpation of cataract and the non-treated cases ?
 I think α -chymotrypsin will possibly have delicate influence on reaction for SH group of protein.
- 2) Could you find any differences in reactions for SH group of protein and in other enzyme activities of lenticular epithelium between the mature cataract and the immature, or between the cases with opaque lenticular epithelium and the cases with clear epithelium ?

Dr. Otori :

- 1) There were not any differences in the reactions between the cases treated with α -chymotrypsin and non-treated cases. But I think it may be impossible to indicate exquisite changes in the lenticular epithelium with the present method.
- 2) The reactions for SH group was completely negative only in the epithelium of brown cataract (or browned parts). But there were no changes in the reactions of any other conditions.

Histochemical Demonstration of Protein-bound Sulfhydryl Groups with Nitro Blue Tetrazolium Salt

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Introduction

Sulfhydryl groups in living cells have been recognized to be related with the keratinizing mechanism and carcinogenic process of the skin, constituting an active centre of enzymes and acetyl Co A.

Histochemical studies for protein bound SH groups also have been carried out by many investigators to the realization of the superiority of D.D.D. and neotetrazolium method for a histochemical detection^{1,2,3)}

Recently, as an excellent tetrazolium salt, nitro blue tetrazolium salt has

been widely employed for the demonstration of many dehydrogenase activities as a hydrogen acceptor and chromogenic agents. In this study, an attempt was made to apply this salt for the demonstration of protein bound SH groups by modifying the routine method of neotrazolium.

A reagent solution of the routine tetrazolium method by Barrnet et al and Pearse which demonstrates both protein-bound sulfhydryl and disulfide groups was kept at high alkaline pH with KCN and NaOH. As a histochemical detection of SH groups, it is preferable to demonstrate SH and SS groups separately.

In the former experiments^{3,4)} it was tried to demonstrate separately SH and SS groups using neotetrazolium chloride in accordance with Gomori's⁵⁾ technique. This reagent solution of Gomori's method was kept at pH 8.6 which happened incapable of reducing disulfide groups, enabled one to obtain more detailed observations than D.D.D-azo dye method.

Reduction of Nitro-BT Under Various pH

In reducing tetrazolium by SH compounds, it is well known that the reduction is closely related with the concentration of KCN and the pH of the reagent solution.⁶⁾ The formation of formazan in a tets tube was accelerated with an increase of pH (Table 1). Although tissue sections showed no significant staining in the solution containing no KCN at pH 8.0~9.2, addition of titrated KCN to this solution gave a good stainability to tissue sections.

Table. 1

The presence of highly concentrated KCN is reasonably considered to result in a reduction of cystine (s-s), and to direct reduction of tetrazolium salt, in a formation of formazan directly from nitro-BT as described by Findley.⁶⁾ Thus if a solution is kept at high alkaline pH with high concentration of KCN, it may often demonstrate sulfhydryl and disulfide groups simultaneously. On the basis of the above mentioned experiments, a histochemical detection of protein bound SH group was attempted. Low alkaline pH of a mixture is to demon-

strate only sulfhydryl group because disulfide groups is never reduced at less than pH 10.2, since titrated KCN accelerates a formation of formazan in tissue sections. It seems ideal to have carried out the reaction at almost neutral pH and at a concentration of KCN less than what is described in the following method.

Material and Method

The tissues obtained from rats oral mucosa, salivary glands, pancreas, liver and some malignant tumors of the human jaw, were prepared in fresh frozen sections in cryostat. Some of them were embedded in paraffin after fixation in trichloroacetic alcohol (80% ethanol containing 1% trichloroacetic acid).

Method

1. Fresh frozen sections or paraffin embedded sections
2. Fixed in trichloroacetic alcohol (100 ml of 80% ethanol + gm of trichloroacetic acid)
3. Sections immersed for 30 ~ 60 minutes at 37°C in a mixture consisting of

0.5 M Borate buffer at pH 8.4	15 ml
M/2M KCN (titrated in buffer solution)	10 ml
Nitro-BT (resolved in buffer solution)	15 mg
4. Washed in acidified water
5. Mounted with balsam

Positive tissues were stained purple red or bluish purple.

Results

Oral mucosa : Epithelial cells in the germinative and parakeratotic layer showed an intense reaction. Stainability in the hornified layer increased as the pH of an alkaline solution was kept at over 11.

Salivary glands : Both acinar and duct cells of the parotid gland and duct cells of the submandibular and sublingual glands were stained moderately. No reactions were observed in the acini of the sublingual gland except for a low staining in peripheral parts of mucinous acini and myoepithelia.

Pancreatic glands : Pancreatic cells reacted more intensely than duct cells. Cells in Langerhans islet were little or nonstained.

Liver : Moderate reactions were observed through the liver cells.

The nucleus : The nuclei of the salivary gland, pancreatic cell and liver cell showed a faint stain, and their staining was variable depending upon the kind of tissues and organs.

Blocking Reaction for Sulfhydryl Group

Performic acid oxidation, and iodide acetate, parachloromercuribenzoate (P.C.M.B.) were employed for SH blocking. Performic acid and iodide acetate showed a complete blocking of the formation of formazan at 30 min. and 6 hrs., respectively. During the present experimentation favorable results were obtained with P.C.M.B.

Discussion

Components in tissue giving rise to a positive alkaline tetrazolium reaction were known to be protein and polypeptide containing cysteine or cystine, reducing sugar and protein, containing sugar, lipid aldehyde, adrenalin and ascorbic acid by Pearse²⁾ and Barrnett and Seligman¹⁾. However, reductants such as free cysteine, glutathione, free reducing sugar, adrenalin and ascorbic acid would fail to react because they would not be present in paraffin sections. Also in fresh prepared sections fixed in 80 % alcohol and washed in water, these reductants would disappear from the sections.

Glucose didn't reduce nitro-BT at low alkaline pH in a test tube examination, and only ascorbic acid rapidly reduced the nitro-BT. The transference of the formazan of nitro-BT into lipid was scarcely or not observed while various dehydrogenases were being demonstrated.

Protein bound sulfhydryl group was considered to take part in the reaction at a low alkaline pH such as pH 8.4, and this low pH was assumed to make a reduction of tetrazolium more specific with protein bound sulfhydryl groups.

Conclusion

1. Histochemical findings of the method employed were almost similar to those of Barrnet-Seligman's method, except for a little better contrast on sections with which the former was credited.
2. The detachment and destruction of sections were protected by the present method which was advantageous in shortenning the reaction time to 1/5-1/10 and in keeping low pH in a solution, over the method in which neoterazolium salts were employed.
3. A fine formazan of nitro-BT gave a strict localization of sulfhydryl groups on tissue sections than neotetrazolium salts.

Reference

- 1) Barrnett, R. J. and Seligman, A. M. : J. Nat. Cancer Inst., 13, c15, 1952.
- 2) Pearse, A. G. E. : J. Histochem. Cytochem., 1, s60, 1u52.
- 3) Deguchi, Y., Okamoto, Y. and Oka, R. : Proc. Jap. Histochem. Ass., 1, 38, 1960.
- 4) Kawakatsu, K., Deguchi, Y. and Takita, Y. : J. Dent. Res., 41, 104, 1962.
- 5) Gomori, G. : Quart. J. Micro. Science, 97, 1, 1956.
- 6) Findley, G. H. : J. Histochem. Cytochem., 3, 331, 1955.

Discussion

- Dr. Mizutani : You must concern yourself about the specificity of the method using tetrazolium salts. Because there are possibilities of nonspecific reduction of tetrazolium salts with many endogenous factors other than -SH groups. From the above-mentioned point of view it seems much more reasonable to pre-treat tissue sections with KCN only rather than to incubate sections in the reaction mixture containing KCN.
- Dr. Deguchi : Yes, we must examine carefully the specificity of the tetrazolium salt method for -SH groups, as you say. It is said, however, that it is impossible for factors except -SH group and Vitamine C to reduce tetrazolium salts at such slight alkaline pH range

as was in this experiment. And moreover, we have used the sensitive tetrazolium salt and KCN at low concentration, and incubation time has been sufficiently short. For the reasons above-mentioned, I think that our method is sufficiently specific for -SH group.

On Histochemical Tests of Unsaturation Bonds of Fatty Acids

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Regarding to other studies, the histochemical tests which probably indicate the existence of unsaturation bonds of fatty acids were critically investigated. In this study human specimen of adrenals were tested. After formal fixation, the sections were cut from the frozen tissues. The experiments and the results are summarized.

(1) Staining reaction by method of Osmium tetra-oxide ;
Chemical mechanism of the blacking by osmium tetroxide is seemed to be relatively clear. In model experiments, blacking reaction of oleic acid was confirmed, the procedure of osmium method is quite simple. Because of these reasons, the staining pattern of osmium method was usually compared with other experiment.

(2) Destruction test for the factor of the tissue sections responsible for osmium tetra-oxide is necessary for this study. Antiformin was found to be (alkaline solution of Sod. hypochlorite) a beneficial reagent for this purpose. As is showed in plate 1 to plate 3, by contact of antiformin solution the factor of osmium staining was destroyed. The action of antiformin can be explained as a halogenization at the site of unsaturation bonds of the fatty acids.

(3) Combine of iodine or bromine at the site of unsaturation bonds are histochemically researched. According to ordinary iodine value test, iodination was performed at the first step.

In the second step, for demonstration of iodine, the present authors used silver picrate ($C_6H_2(NO_2)_3 O Ag, H_2O$) according to the method of G. E. Philbrook and D. J. Massey (J. A. C. S., 73, 3454, 1951).

By this method silver iodide will be formed. Silver iodide was reduced in a hydroquinone solution. The tissue finding is showed in the plate 4. Metallic silver can replace by gold in gold chloride solution as is showed in plate 5 and 6.

In other experiment, bromination was also tried. Thereafter, the bromine was changed in silver bromide. According to photographic technique the silver