Comparative Studies of Histochemical Usefulness of Tetranitro-BT (TNBT) and nitro-BT (NBT)

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In 1962 Tsou et al reported the usefulness of a new tetrazolium salt, Tetra-nitro-BT (2,2',5,5'-tetra-p-nitrophenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene)-ditetrazolium chlovide) as a good reagent for histochemistry of oxidative enzymes.

The authors have demonstrated succinic dehydrogenase system and DPNH diaphorase activity in kidney, cerebellum and Hela cells using TNBT and NBT.

As a result, TNBT was better than NBT in following two items.

- (1) Size of formazan granules of TNBT was more homogeneous and finer than those of NBT.
- (2) Solubility of formazan of TNBT was lower than that of NBT.

Studies of New Buffer Solution in Histochemistry

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In experiments of enzyme reactions, buffer solutions are usually added to the test solution for keeping the hydrogen ion concentration in a certain range. Each constituent of various buffers has its own properties as a chemical

Table 1.

Ac. : 1/10-N Acetic Acid

NTA. : 2Na. 1/10-Mol. Nitrilotriacetic Acid Disodium Salt.

Ac : NTA. 2Na	pH
7 : 3	3.5
6 : 4	3.6
5 : 5	3.7
4 : 6	3.8
3 : 7	3.9
2 : 8	4.0
1 : 9	4.3
0 : 10	7.8

Table 2.

IDA.: 1/10-Mol. Iminodiacetic Acid

IDA.: 2Na. 1/10-Mol. Iminodiacetic Acid Disodium Salt.

IDA : IDA. 2Na	pH	IDA : IDA. 2Na	pH
7 : 3	2.80	4.93 : 5.07	6.80
6.8 : 3.2	3.00	4.90 : 5.10	7.20
6.6 : 3.4	3.15	4.85 : 5.15	7.40
6.2 : 3.8	3.20	4.80 : 5.20	7.65
6.0 : 4.0	3.40	4.75 : 5.25	7.70
5.8 : 4.2	3.50	4.70 : 5.30	7.90
5.6 : 4.4	3.60	4.6 : 5.4	8.00
5.4 : 4.6	3.75	4.5 : 5.5	8.20
5.3 : 4.7	3.80	4.2 : 5.8	8.40
5.2 : 4.8	4.00	4.0 : 6.0	8.60
5.1 : 4.9	4.20	3.8 : 6.2	8.65
5.05 : 4.95	4.60	3.6 : 6.4	8.80
5.03 : 4.97	4.90	3.3 : 6.7	8.90
5.01 : 4.99	5.10	3.0 : 7.0	9.00
5.0 : 5.0	5.40	2.6 : 7.4	9.20
4.97 : 5.03	5.90	2.2 : 7.8	9.30
4.95 : 5.05	6.60	2.0 : 8.0	9.40

Table 3.

Ac.: 1/10-N Acetic Acid.

NTA.: 3Na 1/10-Mol. Nitrilotriacetic Acid Trisodium Salt.

Ac : NTA. 3Na	pH	Ac : NTA. 3Na	pH
7 : 3	4.40	4.9 : 5.1	6.70
6.7 : 3.3	4.50	4.87 : 5.13	7.00
6.0 : 4.0	4.70	4.85 : 5.15	7.30
5.7 : 4.3	4.85	4.82 : 5.18	7.60
5.5 : 4.5	5.00	4.800 : 5.20	7.80
5.3 : 4.7	5.20	4.70 : 5.30	8.01
5.2 : 4.8	5.40	4.60 : 5.40	8.30
5.15 : 4.85	5.50	4.40 : 5.60	8.50
5.1 : 4.9	5.80	4.20 : 5.80	8.70
5.0 : 5.0	6.00	4.0 : 6.0	9.0
4.95 : 5.05	6.30	3.6 : 6.4	9.2
4.93 : 5.07	6.50	3.0 : 7.0	9.4

substance as well as a role for buffer.

We should therefore be careful about other effects of the test solution than a buffer as well in enzyme experiments. In the present study, the authors mixed the solution of acetic acid and its compounds (known as chelating agents) each other and determined the pH value of each mixtures by meter. These mixtures showed an effect of buffer solution to some extent as Walpore's solution does.

The test substances used in this study are as follows, the underlined in

Table 4.

Ac. : 1/10-N Acetic Acid.

EDTA. 4Na. : 1/10-Mol. Ethylenediaminetetraacetic Acid Tetrasodium Salt.

Ac : EDTA. 4Na.	pH	Ac : EDTA. 4Na.	pH
7.0 : 3.0	4.70	4.80 : 5.20	7.60
6.9 : 3.1	5.00	4.75 : 5.25	7.85
6.6 : 3.4	5.20	4.70 : 5.30	8.05
6.4 : 3.6	5.40	4.60 : 5.40	8.20
6.2 : 3.8	5.60	4.50 : 5.50	8.40
6.0 : 4.0	5.80	4.30 : 5.70	8.50
5.8 : 4.2	6.00	4.1 : 5.9	8.70
5.6 : 4.4	6.20	3.9 : 6.1	8.80
5.4 : 4.6	6.40	3.7 : 6.3	8.90
5.2 : 4.8	6.65	3.2 : 6.8	9.05
5.1 : 4.9	6.80	3.0 : 7.0	9.10
5.0 : 5.0	7.00	2.0 : 8.0	9.4
4.9 : 5.1	7.25	0 : 10.0	11.0
4.85 : 5.15	7.45		

Table 5.

EDTA 2Na: 1/10Mol. Ethylenediaminetetraacetic Acid. Disodium Salt.

EDTA 4Na: 1/10 Tetra Sodium Salt.

EDTA 2Na : EDTA 4Na	pH	EDTA 2Na : EDTA 4Na	pH
10.0 : 0	4.4	5.2 : 4.8	7.0
9.8 : 0.2	4.8	5.0 : 5.0	7.4
9.6 : 0.4	5.0	4.8 : 5.2	7.7
9.2 : 0.8	5.2	4.4 : 5.6	8.1
9.0 : 1.0	5.4	4.0 : 6.0	8.4
8.4 : 1.6	5.7	3.6 : 6.4	8.7
8.0 : 2.0	5.85	3.0 : 7.0	9.0
7.4 : 2.6	6.0	2.6 : 7.4	9.1
7.0 : 3.0	6.2	2.4 : 7.6	9.3
6.0 : 4.0	6.5	2.0 : 8.0	9.4
5.4 : 4.6	6.8	0 : 10.0	11.0

parentheses are the abbreviations of each term.

(1) Acetic Acid (Ac) CH_3COOH . (2) Iminodiacetic Acid (IDA), $\text{H.N}(\text{CH}_2\text{COOH})_2$. Disodium Salt (2Na) (3) Nitrilotriacetic Acid (NTA) $\text{N}(\text{CH}_2\text{COOH})_3$. Disodium Salt (2Na) Trisodium Salt (3Na) (4) Ethylenediaminetetraacetic Acid (EDTA) $(\text{HOOC} \cdot \text{CH}_2)_2 \text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{N}(\text{CH}_2\text{COOH})_2$ Disodium Salt. (2Na), Tetrasodium Salt (4Na).

The pH values of mixtures are shown in the following five tables.

The usefulness of these solutions in enzyme experiments was proved for the peculiar purposes. For instance (1) Phytin is a derived substance from seeds and may probably be inositol hexaphosphonic acid salt. $(\text{C}_6\text{H}_6\text{P60}_2\text{Ca}_5\text{Mg}_4)$. This is soluble in acid but insoluble in alkali.

Using the new buffer solution (Table 5, EDTA solution) such substance as phytin will be tested in alkaline media as a substrate of enzyme action, (2) If this buffer is used, we can try histochemical azo-dye method under complete elimination of the influence of magnesium and of many other metals. Studies using these buffer solutions for the enzyme experiment will be reported by the present authors at the next meeting.

Some Comments on the Section Freeze-Substitution Technique

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Freeze substitution originally described by Simpson in 1941 (*Anat. Rec.* 80: 173) differs from freeze drying in some respects: first, it needs only cold baths and boxes, instead of requiring an expensive vacuum system. Second, tissues can be exposed to fixation during the dehydration process. The disadvantages are: it requires several days for dehydrating tissues of a few millimeter thickness, and inactivates a number of enzymes during the process, as in the case of freeze drying.

To obviate these disadvantages, Chang and Hori devised a modified technique named "Section Freeze-Substitution Technique", the details of which were published elsewhere (*J. Histochem.* 9: 292; 10: 592; *Ann. Histochem.* 6: 419). Its principle is the dehydration of frozen sections (but not frozen pieces of tissues) in acetone at dry ice temperature. With this technique a number of both oxidative and hydrolytic enzymes and non-enzyme substance have been readily and precisely demonstrated in tissue sections.

In the following are presented some comments on this technique, the demonstration of lipids and adenosine triphosphatase activity in cytoplasm, and the application of this technique to electron histochemistry.

1. Substitution solvent: The substitution solvents so far tested were methanol, ethanol, butanol and acetone. All the solvents afforded good preservations of the cellular morphology, but the former two were not adequate for the study of enzymes, since they were inhibitory for some enzymes. Butanol was less harmful to certain enzymes than acetone, but it became viscous at dry ice temperature, so that the addition of ethyl chloride was required in order to lower the eutectic point and make it maneuverable. For these reasons, acetone was chosen as an all-purpose substitution solvent.

2. Celloidin coating and demonstration of ATPase activity in cytoplasm: Celloidin coating at dry ice temperature is a necessary procedure for localization