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Histochemical Method for Demonstrating Aconitase.

アコニターゼの組織化学的証明法.

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Aconitase is an enzyme which is responsible for the interconversion of cis-aconitic, isocitric and citric acid by either hydration and dehydration or intramolecular rearrangement (c. f. DICKMAN 1961). It is widely distributed as a member of the TCA cycle system of enzymes in plant as well as in animal tissues. In the animal it is particularly abundant in muscle, liver, kidney and in prostate (c. f. OCHOA 1951).

Histochemical methods for aconitase itself have not been reported as yet, because of the difficulty of applying direct test of the activity to histochemistry. In order to stain TPN-diaphorase with tetrazolium, FARBER et al. (1954) used citrate as starting substrate, which was said to be converted to isocitrate by tissue aconitase. We also found that OCHOA's indirect method using isocitric dehydrogenase and TPN (1951) can be successfully combined with tetrazolium reduction (NACHLAS et al. 1957). The present report includes the description of the method, brief notes of the enzyme distribution, specificity and limitation of the method.

I. Materials and Methods.

Materials. The organs used included the liver, kidney, brain and small intestine of rat, and the prostate of man and dog. The materials of rat were cut within 30 minutes after killing the animal. The materials from man and dog were quickly frozen on solid CO₂ after the removal and stored for 2 or 3 days. The sections were cut at 30 μ thickness with a cryostat and dried for 2 to 4 hours at room temperature.

Methods. The test is based on the formation of isocitric acid from cis-aconitic or citric acid in the presence of aconitase. Isocitric acid formed is then dehydrogenated by isocitric dehydrogenase present in the section with the formation of reduced TPN, which can reduce nitro-BT to form formazan by TPN-diaphorase. As the rate of TPN reduction is about 4 times faster with cis-aconitate than with citrate as substrate (OCHOA 1951), we usually used cis-aconitate as substrate. Since Mn⁺⁺ is the activator of isocitric dehydrogenase, it was added to the incubating solution to promote the reaction sequence and to gain the deeper coloration.

The standard incubating solution is composed of the following reagents.

1. 0.2 M Sodium cis-aconitate	4 ml
2. 0.005 M Manganese chloride	2 ml
3. TPN (triphosphopyridine nucleotide)	5 mg
4. Nitro-BT	10 mg
5. 0.05 M Veronal buffer (pH 7.4)	10 ml
6. Distilled water	4 ml

Sodium salt of cis-aconitic acid was prepared as follows: 348 mg of cis-aconitic acid was dissolved in 4.0 ml of distilled water and 1.5 N NaOH was added to make pH 7.0. Then the solution was made total volume of 10.0 ml with distilled water (final concentration is 0.2 M).

In order to *test the specificity* of the method, several variations of the mixture were examined.

- A. Omitted sodium cis-aconitate from the complete mixture.
- B. Omitted TPN.
- C. Omitted manganese chloride.
- D. Aconitate was replaced by the same concentration of sodium citrate.
- E. Aconitate was replaced by the same concentration of sodium isocitrate.
- F. Added 2 ml of 0.1 M KCN (aconitase inhibitor) to the complete mixture*.
- G. Added 2 ml of 0.1 M KCN to the test E*.
- H. TPN was replaced by the same concentration of DPN.
- I. α, α' -dipyridyl (aconitase inhibitor) (= DP) was tested by either preincubation with it or adding it to the complete mixture.
- J. *p*-chloromercuribenzoic acid (isocitric dehydrogenase inhibitor) (= PCMB) (LOTSPEICH and PETERS 1951) was tested in the same way as I.

The sections were incubated in these mixtures for 30 to 180 minutes at 37°C. Then they were post-fixed in 10 % neutral formalin for 12 to 24 hours and mounted in canada balsam after dehydration through the gradual alcohol and xyrol.

II. Results.

The deposition of formazan in the present test is ascribed to the activity of not only aconitase but also isocitric dehydrogenase and TPN diaphorase. Accordingly the method may be regarded as the histochemical test for aconitase-isocitric dehydrogenase system. The following is the brief description of the activity in some organs.

Kidney: An intense coloration was observed in the epithelial cells of the proximal convoluted tubules, and a moderate one in those of the thin portion of HENLE's loop. The distal convoluted tubules exhibited a weak reaction. The thick portion of HENLE's loop and glomerulus reacted faintly. The blood vessels were negative (Fig. 1).

Small intestine: A moderate to strong reaction was shown in the epithelium, and a weak one was in the lamina propria and tunica muscularis.

Liver: An intense reaction occurred in the hepatic cells especially of the peripheral part of each lobule.

* In these cases, the sections were preincubated in 0.01 M KCN solution for 30 minutes at room temperature.

Fig. 1. Aconitase activity of the kidney of rat. The proximal convoluted tubules exhibit strong activity. The glomerulus and distal convoluted tubules are faintly reactive. The inner cortex shows little coloration. In the outer and inner medulla, a moderate reaction is demonstrated in the thin portion of HENLE's loop. $\times 16$

Fig. 2. Prostate of man (hypertrophic). Cytoplasm of the glandular cells are deeply stained. Connective and muscular tissue stroma exhibits a weak reaction. Note the heterogeneous coloration in the glandular cells. $\times 120$

Brain: The intracerebral blood capillaries were moderately to strongly reactive, and the nerve cells exhibited a faint reaction. The nerve fibers were almost negative with some exceptions.

Prostate of man and dog: Glandular cells of prostate of both species were markedly stained, while the muscular and connective tissue stroma exhibited a weak to moderate reaction. But in the prostatic cancer, the stroma showed somewhat increased activity. In the typical hypertrophy of the prostate, the glandular cells lining the enlarged lumen exhibited a reduced activity (Fig. 2).

Specificity tests:

- A. Without sodium cis-aconitate, no coloration occurred.
- B. When TPN was omitted, no reaction was observed.
- C. In the absence of manganese chloride, the reaction was markedly suppressed.
- D. When aconitate was replaced by sodium citrate, the coloration became much fainter.
- E. Although aconitate was replaced by sodium isocitrate, the coloration was as strong as in the complete mixture.
- F. When the section was treated with KCN, no reaction was observed.
- G. When isocitrate was used as substrate and the section was treated with KCN, no inhibition was observed. The intensity of the reaction was as high as in the case of the complete mixture.
- H. When TPN was replaced by DPN, no reaction could be seen.

I and J. Results of experiments I and J are summarized in Table I.

Table 1. Action of DP and PCMB on Aconitase and Isocitric Dehydrogenase.

Substrate	Inhibitor		Activity, Coloration
	Preincubation	Incubation mixture	
Aconitate	non	non	+++
	non	DP	±
	non	PCMB	—
	DP	DP	—
	PCMB	PCMB	—
Isocitrate	non	non	+++
	non	DP	++
	non	PCMB	+
	DP	DP	±
	PCMB	PCMB	—

The concentration of DP was 0.001 M but that of PCMB was inconstant because of being hardly soluble. In the preincubation each inhibitor was dissolved in the buffer solution and acted upon the section for 30 minutes at room temperature.

Table 1 indicates that DP inhibits aconitase more strongly than isocitric dehydrogenase, and isocitric dehydrogenase is inhibited by PCMB more markedly than by DP.

From the specificity tests described above it became evident that the coloration is ascribed to the activity of aconitase with concomitant isocitric dehydrogenase. With

the use of inhibitors and activator and by changing substrate one can identify the individual enzyme activity with certainty.

III. Discussion.

In their technique on the histochemical demonstration of TPN diaphorase, FARBER et al. (1954) used the tissue aconitase activity employing citrate as substrate to produce isocitrate. The results of our experiments also showed that the coloration of tissue is brought out by the sequence of the following reaction:

Aconitate is now regarded as not an obligatory intermediate but rather a side-product with a common intermediate (FRIEDLICH-FRESKA and MARTIUS 1951, SPEYER and DICKMAN 1956). However, since the reaction (1') proceeds faster than the reaction (1) (OCHOA 1951, Test D), aconitate is more favourable substrate for aconitase activity. Moreover, the equilibrium constants between citrate, cis-aconitate, and isocitrate are 90.9% : 2.9% : 6.2% (pH 7.4, 25°C) (KREBS 1953), but when cis-aconitate is used as substrate, the conversion to citrate and isocitrate proceeds at approximately the same verocity (MARTIUS and LEONHARDT 1943). In the presence of isocitric dehydrogenase isocitrate is converted to oxalosuccinate as soon as it is formed. As the accumulation of isocitrate cannot occur under such condition as this method, the reaction (1') proceeds from left to right, and most of aconitate is utilized as substrate. On the other hand, the formation of isocitrate from citrate is much slower in rate and therefore the reduction of TPN might be slowed. In fact, the coloration of tissue in the case of test D was remarkably weak even after incubation for 3 hours.

Although aconitase is activated by Fe^{++} (DICKMAN and CLOUTIER 1950, MORRISON 1954), it is difficult to use Fe^{++} in this method, because this ion itself reduces nitro-BT. On the other hand, Mn^{++} , the activator of isocitric dehydrogenase (ADLER 1939, OCHOA and WEITZ-TABORI 1945, SIEBERT et al. 1957), can be added to yeild a deeper colcration (Test C). It is said that the enzyme does not require Mn^{++} for the dehydrogenase reaction (OCHOA 1945, MOYLE 1956) but for the decarboxylation of oxalosuccinate to α -ketoglutarate (FARBER et al. 1954, KREBS and LOWENSTEIN 1960). Anyway, the two reactions are linked so closely that one reaction cannot proceed unless the other occurs (MOYLE 1956). Aconitase is inhibited by iron-binding agents such as cyanide (Test F) and DP (Test I), but isocitric dehydrogenase is not inhibited by cyanide (Test G) and rather sensitive for PCMB (Test J).

We used veronal buffer, for phosphate inhibits isocitric dehydrogenase perhaps by precipitating Mn^{++} (LOTSPEICH and PETERS 1951). Tris buffer was also useful.

In the quantitative investigation BARRON and HUGGINS (1946) have reported considerable accumulation of isocitric acid in the hypertrophic prostate of man and

they explained this fact by high activity of aconitase. In fact, they demonstrated that aconitase activity of hypertrophic prostate of man is about 2 times higher than that of normal dog. However, our result showed that the typical hypertrophic prostate is rather low in aconitase activity than normal dog. On the other hand, the results of rat liver and kidney well agree to JOHNSON's report (1939).

Since this method involves isocitric dehydrogenase and TPN-diaphorase besides aconitase, the obtained results must be compared with that of test E, isocitric dehydrogenase. If some tissues contain isocitric dehydrogenase but not aconitase, the difference of histochemical findings following two methods should be evident. Up to the present time we have not found any differences between the two activities. Therefore aconitase is thought to be generally co-exist with isocitric dehydrogenase. However, the cells and tissues should contain only aconitase without concomitant isocitric dehydrogenase, the present method fails to bring out the aconitase. In this case we could demonstrate the activity by adding the isocitric dehydrogenase preparation to the mixture.

IV. Summary.

1. A method for histochemical demonstration of aconitase was presented based on OCHOA's indirect method using isocitric dehydrogenase and TPN combined with nitro-BT reduction.

2. The incubation medium was composed of 4 ml of 0.2 M sodium cis-aconitate, 10 ml of 0.05 M veronal buffer (pH 7.4), 2 ml of 0.005 M manganese chloride, 5 mg of TPN, 10 mg of nitro-BT, and 4 ml of distilled water.

3. The liver, kidney, small intestine and brain of the rat, and the prostate of man and dog were investigated with this method. Strong activity was demonstrated in the hepatic cells of the peripheral part of the hepatic lobules, in the epithelial cells of the proximal convoluted tubules of the kidney, and glandular cells of the prostate.

4. The method is examined with respect to the specificity of enzyme activity concerned by adding or omitting substrates, cofactors, activators and inhibitors to or from the mixture. It is clear that the method demonstrates aconitase with co-existent isocitric dehydrogenase and TPN-diaphorase. DPN-linked isocitric dehydrogenase might not be concerned in this method.

内 容 自 抄

1. アコニターゼの組織化学的検出法を、イソクエン酸脱水素酵素とトリホスホピリジンスクレオチドを使用する Ochoa の間接証明法とニトロブリューテトラゾリウムの還元法を結合することにより、考案した。

2. 温置混合液の組成は次の通りである。

0.2 M sodium cis-aconitate	4 ml
0.05 M veronal buffer (pH 7.4)	10 ml
0.005 M manganese chloride	2 ml
TPN	5 mg
nito-BT	10 mg
distilled water	4 ml

3. 本法によりラットの肝臓、腎臓、小腸、大脳、また人と犬の前立腺を検索したところ、強い反応が特に肝小葉周縁部の肝細胞、腎尿細管主部(曲部)の上皮、前立腺の腺上皮等に見られた。

4. 本反応の特異性を、基質、補酵素、促進剤、阻害剤を温置混合液に加え、または除いて、検討し、本法はアコニターゼ及びそれと共存するイソクエン酸脱水素酵素とトリホスホピリジンスクレオチド-ジアホラーゼをよく証明する事を確実にした。しかしジホスホピリジンスクレオチド連鎖のイソクエン酸脱水素酵素は干渉していない。

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