medium and the so called specificity of acid phosphatase. Prof. S. Akamatsu's opinion must be highly estimated. Though we were not able to admit that phosphamidase in a cancer cell was specific.

We tried with the Takamatsu and Nishi's⁹⁾ method, through alkaline medium using muscle adenylic acid as substrates but the reaction proved to be not specific. As phosphoric acid is split from these acids by the action of nonspecific alkaline phosphatase, without demonstrating of special enzyme activity on adenylic acids, the tissues may be stained by nonspecific enzyme. So we cannot admit the specificity in such a experiment.

We tried to demonstrate Adenyl-pyrophosphatase¹³⁾ (H. Takamatsu and K. Iijima 1942) and Nucleophosphatase (phosphatase for relative depolymerized nucleic acid) (H. Takamatsu and H. Izaki 1952)¹⁴⁾ in cancer tissue but we were not able to admit the specific reaction.

References

- 1) H. Takamatsu; Journ. Orient. Med. (Manshu Igaku Zashi) **23**, 1351, 1938. 2) H. Takamatsu; Trans. Soc. Path. Jap. **29**, 492, 1939. 3) H. Takamatsu; Tokyo Ijishinshi. No. 3161. 1939. 4) H. Takamatsu; "Gan" **33**, 218, 1939. 5) H. Takamatsu; "Gan" **34**, 81, 1940. 6) H. Takamatsu, and T. Otuki; "Gan" **35**, 283, 1941. 7) T. Otuki; Report 1-5; Manshuigaku Zashi, vol. **36**, **37**, **38**, 1942-1943. 8) H. Takamatsu and H. Nishi; Trans. Soc. Path. Jap. **43**, Ed. gen, 546, 1954. 9) H. Takamatsu and A. Shinoto; Trans. Soc. Path. Jap. **41**, Ed. gen, 1952. 10) A. Shinoto; The Yokohama Med. Jour. **3**, no 3, 1952. 11) H. Takamatsu, S. Ozawa and H. Izaki; the Digest. Int. & For. Lit. Hist. path. 1, 1951. 12) H. Takamatsu and E. Sho; Trans. Soc. Path. Jap. **32**, 90, 1942. Manshu Igaku Zashi, vol. **36**, Supl. 1942. Ibid. vol. **36**, 993, 1942. **38**, 581, 761, 901, 1943. 13) H. Takamatsu & K. Iijima; Manshu Igaku Zashi, **36**, Supl. 1942. 14) H. Takamatsu & H. Izaki; Trans. Soc. Path. Jap. (Ed. Reg.) **41**, 1952.

非特異的脱磷酸酵素と ATP 脱磷酸酵素の組織化学的 鑑別, 特に酸性 ATP 脱磷酸酵素について

前川孫二郎・木村俊二・中本公明・池田碩二

京都大学医学部内科第三講座

Histochemical Differentiation of Non-Specific Phosphatases and ATP-Phosphatases with Special Reference to Acid ATP-Phosphatase

Magojiro MAEKAWA, Shunji MOTOMURA, Kimiaki NAKAMOTO and Seiji IKEDA

The Third Medical Clinic, Kyoto University Hospital

The first histochemical demonstration of alk. ATP-ase was attempted by Takamatsu¹⁾. It was further investigated by many workers with paraffin and cryostat sections^{2,3,4,5,6)}. Recently, many of the difficulties and uncertainties

in the histochemical demonstration of alk. ATP-ase were clarified by Padykula and Herman⁷⁾, Novikoff⁸⁾, and Freiman and Kaplan⁹⁾. While the histochemical demonstration of ATP-ase at a physiologic pH was advocated by Wachstein and Meisel¹⁰⁾, that of ac.ATP-ase has not so far been reported. The specificity of the histochemical demonstration of ATP-ase in spite of previous extensive studies by many investigators, has not as yet been decidedly established. Therefore, by applying the Takamatsu-Gomori principle, the histochemical demonstration of ac.ATP-ase was undertaken and investigations of the specificity of the method were made with the paraffin sections in which either non-specific phosphatase or ATP-ase was inactivated by pretreatment with heated water.

Material and Methods

I. Preparation of sections

Rabbits and albino rats were killed by air embolism and the organs were rapidly removed. Tissues were fixed and dehydrated in 4 to 5 changes of a ice-cold acetone-alcohol mixture within 24 hr. The tissues were cleared in 3 changes of xylol within 45min. and embedded in paraffin at a temperature lower than 54°C within 60min. Sections were cut at 10 μ and placed on uncoated cover glasses.

II. Preparation of incubating media and procedures of staining

1) Incubating medium for acid ATP-ase

A	{ M/5 acetate buffer (pH: 5.5):	
	2.0cc 2% lead nitrate	: 0.6cc (final concentration: 0.0036M)
B	{ ATP-2Na	
	distilled water	: 10mg (final concentration: 0.0016M) : 6.0cc

2) Incubating medium for alkaline ATP-ase

A	{ M/10 sodium barbiturate	
	3% calcium chloride	: 4.0cc : 1.0cc (final concentration: 0.0204M)
B	{ ATP-2Na	
	distilled water	: 20mg (final concentration: 0.0032M) : 4.0cc

The pH of the solution B was adjusted to 7.0 with 1N sodium hydroxide, poured into the solution A and stirred. The pH of the mixture was adjusted to 5.5 in the ac.ATP-ase medium and to 9.2 in the alk.ATP-ase medium with 1N sodium hydroxide or 1N hydrochloric acid and made up to 10.0cc with distilled water. The molar concentration of ATP-2Na shown above is a calculated value. According to Kano¹¹⁾, the actual concentration of ATP-2Na in the filtrate of the mixtures determined by Chon and Carter's method was by 5 to 10% lower than that calculated. But when the solution A was poured into the solution B, the turbidity of the mixture was marked and a decrease in the ATP concentration in the filtrate was more marked. The ATP solution has been thought to be unstable. According to Kano, however, ATP-2Na in the solutions adjusted to a pH 5.5 with acetate buffer and to a pH 9.2 with barbiturate buffer did not decrease, even when they were kept in a 37°C oven for 4 hours. Therefore, there is no possibility for unenzymatic hydrolysis of ATP

to interfere enzymatic histochemical reactions during incubation periods which are never longer than 180 minutes.

After deparaffinization, sections were incubated in the media in a 37°C oven for 15 to 180min., depending on the activity of the tissue studied and washed well under the tap water for 5 min. The sections incubated in the ac. ATP-ase medium were immersed in 2% yellow ammonium sulfide for 5 min. But the sections incubated in the alk.ATP-ase medium were transferred to 2% cobaltous chloride for 5 min. before being immersed in 2% yellow ammonium sulfide. The sections were then washed well under the tap water for 5 min., dehydrated in 80%, 95%, 100% ethanol, cleared in xylol and mounted in Canada balsam. Non-specific acid and alkaline phosphatase were demonstrated by Takamatsu's and Gomori's method which was slightly modified by us.

Results

In the tissue cells specific to the organ, the distribution pattern of ac. ATP-ase was similar to that of non-specific ac. P-ase and that of alk. ATP-ase was also similar to that of non-specific alk. P-ase in all the organs studied (the brain, thyroid, lung, liver, spleen, kidney, intestine, pancreas, and adrenal) with the exceptions of the hypophysis and islets of Langerhans of the pancreas. Namely, non-specific ac. P-ase was found mainly in the Pars intermedia and Pars nervosa of the hypophysis and in the β -cells of the islet of Langerhans. While, ac. ATP-ase was found mainly in the Pars distalis of the hypophysis and in the α -cells of the islet of Langerhans. Common difference in the distribution of non-specific phosphatase and ATP-ase both acid and alkaline in all the organs studied was the behaviors of the nuclei, erythrocytes, blood vessels and the intestinal, cardiac and skeletal muscles. Namely, nuclear staining did not occur in the ATP-ase media except in nerve cell bodies and ganglion cells. Ac.ATP-ase was found in the erythrocytes, the media of vascular walls, muscular layers of intestinal walls and the cardiac and skeletal muscles. Alk. ATP-ase was found mainly in the intima and adventitia of blood vessels. However, the distribution of ATP-ases in vascular walls showed some difference according to the size, species and the organ to which they belong. For example, alk. ATP-ase which was usually absent in the media of blood vessels was intensely present in that of the coronary artery and the adventitia which failed to react in the most rabbit organs was heavily stained in the most rat organs in the alk. ATP-ase medium.

Specificity

As described above, the distribution patterns of ac. and alk. ATP-ase in the tissue cells specific to the organ were similar to those of ac. and alk. P-ase respectively with a few exceptions. In order to ascertain this similarity in the non-specific P-ase and ATP-ase distribution patterns, the following tests were performed.

I. Pretreatment of sections with heated water

Basing on our previous report that ac. P-ase in a ac. ATP-ase fraction of dog kidney was inactivated by keeping it at 56°C for 30 min. without any decrease in ac. ATP-ase¹²⁾, deparaffinized sections were immersed for 30 min. in water regulated at the temperatures tested by a thermostat. As shown in table 1, the stabilities of non-specific P-ase and ATP-ase against heated water differed according to the organ and species studied. But in all the organs studied, ac. ATP-ase was more stable than non-specific ac. P-ase and non-specific alk. P-ase more than alk. ATP-ase. And of all the enzymes studied, ac. ATP-ase was the most stable against heated water.

Table 1 Activities of P-ases and ATP-ases after Pretreatment with Heated Water

A. Rabbit Liver																	
Temperature (°C)		56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	
acid	P-ase	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	
	ATP-ase	++	++	++	++	++	++	++	++	++	++	++	++	++	+	-	
alk.	P-ase	++	++	++	++	++	++	++	++	+	-	-	-	-	-	-	
	ATP-ase	++	+	+	+	+	+	+	+	-	-	-	-	-	-	-	

B. Rabbit Intestine																	
Temperature (°C)		56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	
acid	P-ase	++	++	++	++	++	++	++	++	+	-	-	-	-	-	-	
	ATP-ase	++	++	++	++	++	++	++	++	++	++	++	++	++	+	-	
alk.	P-ase	++	++	++	++	++	++	++	++	++	++	++	++	++	+	-	
	ATP-ase	++	++	++	++	++	++	++	++	++	++	++	++	++	+	-	

C. Rat Kidney																	
Temperature (°C)		56	58	60	62	64	66	68	70	72	74	76	78	80	82	84	
acid	P-ase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	ATP-ase	++	++	++	++	++	++	++	++	++	++	++	++	++	+	-	
alk.	P-ase	++	++	+	-	-	-	-	-	-	-	-	-	-	-	-	
	ATP-ase	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	

The amount of reaction products occurred in the sections 66°C-treated and untreated is shown in table 2.

Non-specific ac. P-ase was completely inactivated by the pretreatment with heated water but ac.ATP-ase remained almost completely unaffected. In the sections of rat kidney in which non-specific alk.P-ase was completely inactivated, no reactions occurred also when incubated in the alk.ATP-ase medium. But in the sections of such organs as rabbit, kidney and liver in which non-specific alk. P-ase remained almost unaffected, a trace of reaction products

Table 2. Activities of P-ases and ATP-ases in the Sections 66°C-treated and untreated

	acid				alkaline			
	P-ase		ATP-ase		P-ase		ATP-ase	
	66°C	untr.	66°C	untr.	66°C	untr.	66°C	untq.
Rat Kidney	—	##	##	##	—	##	—	##
Rabbit Kidney	—	##	##	##	##	##	+	##
Rabbit Liver	—	##	##	##	##	##	+	##

occurred also when incubated in the alk. ATP-ase medium. The distribution pattern of ac. ATP-ase in the 66°C-treated sections was completely the same as in the untreated sections as shown in figures 1 and 2. The distribution patterns of reaction products in the 66°C-treated sections of such organs as rabbit liver and kidney were completely the same as those in the untreated sections when they were incubated in the non-specific alk.P-ase medium. But when they were incubated in the alk. ATP-ase medium, the amount of reaction products in the 66°C-treated sections was markedly small when compared with that in the untreated sections and the distribution of this small amount of reaction products corresponded to the sites where non-specific alk. P-ase in the 66°C-treated sections showed the most intense activity as shown in figures 3 and 4. Accordingly, a trace of reaction products in the 66°C-treated sections occurred in the alk.ATP-ase medium is supposed to be due to a non-specific dephosphorylation of ATP by non-specific alk. P-ase and the enzyme which was inactivated by the pretreatment with 66°C water is supposed to be alk. ATP-ase. Consequently, the reaction products in the untreated sections occurred in the alk. ATP-ase medium ought to be due to a specific dephosphoryla-

Fig. 1 Rabbit liver, 66°C treated, incubated in the acid ATP-ase medium for 180 minutes. $\times 400$.

Fig. 2 Rabbit liver, untreated, incubated in the acid ATP-ase medium for 180 minutes. $\times 400$.

The distribution pattern of acid ATP-ase in the 66°C treated section is the same as that in the untreated. Strong cytoplasmic staining without nuclear staining is seen.

Fig. 3 Rabbit liver, 66°C treated, incubated in the alkaline ATP-ase medium for 120 minutes. $\times 100$.

Fig. 4 Rabbit liver, 66°C treated, incubated in the non-specific alkaline P-ase medium for 5 hours. $\times 100$.

The site of a trace of reaction products in the alkaline ATP-ase medium corresponds to the site where the heaviest staining occurs in the non-specific alkaline P-ase medium.

Fig. 5 Rat kidney, untreated, incubated in the acid ATP-ase medium for 180 minutes. $\times 400$.

Fig. 6 Rat kidney, untreated, incubated in the alkaline ATP-ase medium for 120 minutes. $\times 400$.

Acid ATP-ase in the media and alkaline ATP-ase in the adventitia of vascular walls.

tion of ATP by alk. ATP-ase.

As shown in table 3, the non-specific dephosphorylation of ATP by alk. P-ase in the alk.ATP-ase medium could be prevented by shortening the incubation periods.

A sufficient amount of reaction products due to a specific dephosphorylation of TAP by ATP-ase occurred before the reaction products due to a non-specific dephosphorylation of ATP by non-specific P-ase became visible. Therefore, the reaction products in the sections incubated in the ATP-ase media can be said

Table 3 Reaction Products and Incubation Periods in alk. ATP-ase Medium

		35min.		70min.		120min.	
		66°C	untr.	66°C	untr.	66°C	untr.
Reaction Products	Rabbit Liver	—	++	—	##	+	##
	Rabbit Kidney	—	++	—	##	+	##

to be caused by a specific dephosphorylation of ATP-ases.

II. Pretreatment of sections with heated air

It is a well known fact that the heat stability of fresh undehydrated sections is low. Our pretreatment was performed in water on the deparaffinized sections once fixed and dehydrated. In order to exclude the influence of water, pretreatment was performed with heated air on the sections not deparaffinized. The activities of P-ase and ATP-ase of both acid and alkaline were not reduced at all even when the temperature was kept at 82°C for 30min. It seems that the activities of P-ases and ATP-ases are completely protected against heat in the absence of water within paraffin until the enzyme proteins were denatured.

III. The effect of phenothiazine derivatives on the staining reactions

Chlorpromazine (0.0014M) completely inhibited non-specific ac. P-ase and slightly activated ac. ATP-ase. Mepazine (0.016M) moderately inhibited non-specific ac. P-ase and moderately activated ac.ATP-ase. Therefore, phenothiazine derivatives such as chlorpromazine and mepazine can be used routinely for the specific histochemical demonstration of ac. ATP-ase as sulfhdrdryl compound (BAL and cysteine) for that of alk.ATP-ase recommended by Padykula and Herman. Non-specific alk. P-ase was slightly activated and alk. ATP-ase was moderately inhibited by these phenothiazine derivatives.

IV. The effect of magnesium ion on the staining reactions

Magnesium ion(0.0025M)activated P-ase and ATP-ase of both acid and alkaline. Therefore, the histochemical differentiation of non-specific P-ases and ATP-ases by magnesium ion was impossible. However, the degree of activation by magnesium ion was the highest on non-specific ac.P-ase and the lowest on alk. ATP-ase.

V. The effect of synthetic adrenocortical hormones on the staining reactions

The influences of 0.0012M cortisone acetate and 0.0014M prednisolone differed according to the organs studied. Dexamethasone (16-methyl-9-fluoroprednisolone) (0.00025M) inhibited ac. ATP-ase markedly and alk. ATP-ase moderately. However, the effects of the solvents of these drugs alone were not examined.

Discussion

If apyrase or polyphosphatase which is said to be able to utilize ATP as substrate is a single enzyme, it must be consisted of a different protein with

different stability against the pretreatment with heated water. And this difference of the enzyme in the stability against heated water must have been reflected in the stained sections as a difference in the distribution pattern and amount of reaction products before and after the inactivation of the enzyme concerned, unless the low rate of splitting ATP by these less specific enzyme failed in producing as much reaction products as visible in limited incubation periods. The histochemical identification and differentiation of the enzyme have so far been made by comparing the distribution pattern and amount of the reaction products occurred in the presence or absence of inhibitors or activators with or without pretreatment of sections. In this respect, it can be stated that in each medium there is one enzyme which dephosphorylates ATP in our ATP-ase media.

Freiman and Kaplan, demonstrating ATP-ase histochemically by the methods of Padykula-Herman and Wachstein-Meisel with or without cation pretreatment, indicated the presence of another enzyme with ATP-ase in intestinal smooth muscle and divided the ATP-dephorylating enzymes into three; a highly specific enzymes capable of splitting only ATP and inosine triphosphate, a non-specific alk. P-ase, and a polyphosphatase of lower specificity which is largely present in small blood vessels and intestinal smooth muscle. The pH of the media used by them ranged between 7.2 and 9.4 and the presence of the enzyme which has its optimum pH in acidity was not taken into consideration.

Novikoff, Hansman and Podber, applying a biochemical determination of ATP-ase in homogenates and a histochemical demonstration of ATP-ase in isolated subcellular fractions and sections of rat liver, pointed out the difference in the behaviors of the ATP-ase of the bile canaliculi and those of the mitochondria and indicated the possibility of different enzymes. Their investigations were also made with the media of Padykula-Herman and Wachstein-Meisel and the presence of ac. ATP-ase was not considered.

In our experiments, ac. ATP-ase was found in the mitochondria of hepatic cells, media of vascular walls and muscular layers of intestinal walls, and alk. ATP-ase in the intima and adventitia of vascular walls and bile canaliculi.

Biochemical determination of the effects of phenothiazine derivatives on ATP-ase were also made with neutral or alkaline media and their stimulating effect on ac. ATP-ase was not noticed^{13,14}.

Summary

Ac. ATP-ase was histochemically demonstrated by applying the Takamatsu-Gomori principle. In the tissue cells specific to the organ, the distribution pattern of ac. ATP-ase was similar to that of non-specific ac. P-ase and that of alk. ATP-ase to that of non-specific alk. P-ase with the exceptions of the hypophysis and islets of Langerhans. Ac.ATP-ase was found in the erythrocytes, media of vascular walls, muscular layers of intestinal walls and the cardiac and skeletal muscles. Non-specific P-ase and ATP-ase both acid and alkaline in paraffin sections showed different stability against heated water,

ac. ATP-ase being more stable than nonspecific ac. P-ase and non-specific alk. P-ase more than alk. ATP-ase. Chlorpromazine or mepazine can be used routinely as the inhibitor of non-specific ac. P-ase and the activator of ac. ATP-ase. Dexamethasone inhibited ac. ATP-ase markedly and alk. ATP-ase moderately.

Acknowledgment

We wish to express our thanks to Prof. H. Takamatsu and the staff of his laboratory for giving us the freedom and every opportunity of receiving guidance and assistance for this investigation.

References

- 1) Takamatsu, H., Iijima, K. and Karasawa, S.: Manshu-Igakukaishi, Memorial Issue for Prof. Kubo, 215-231, 1942.
- 2) Glick, D. and Fisher, E. E.: Science, 102, 429-430, 1945.
- 3) Wang, K. J.: Chinese J. Physiol., 17, 317-324, 1950.
- 4) Maengwyn-Davies, G. D., Friedenwald, J. S. and White, R. T.: J. Cell. Comp. Physiol., 39, 395-447, 1952.
- 5) Takeuchi, T. and Ohkawara, T.: Medicine and Biology, 26 (6), 224-226, 1953.
- 6) Takeuchi, T. and Ohkawara, T.: Medicine and Biology, 28 (3), 123-126, 1953.
- 7) Padykula, H. A. and Herman, E.: J. Histochem. Cytochem., 3, 161-169 and 170-195, 1955.
- 8) Novikoff, A. B., Hausman, D. H. and Podber, E.: J. Histochem. Cytochem., 6, 61-71, 1958.
- 9) Freiman, D. G. and Kaplan, N.: J. Histochem. Cytochem., 8, 159-170, 1960.
- 10) Wachstein, M. and Meisel, E.: Am. J. Clin. Path., 27 (1), 13-23, 1957.
- 11) Kano, K. (Kowa Pharmaceutical Co., Nagoya), personal communication, 1960.
- 12) Maekawa, M., Motomura, S., Ikeda, S., Nakamoto, K., and Aoyama, S.: Tonyobyō (Diabetes), 3, 101-102, 1960.
- 13) Aboud, L. G.: Proc. Soc. Exper. Biol. Med., 88, 688, 1955.
- 14) Century, B. and Horwitt, M. K.: Proc. Soc. Exper. Biol. Med., 91, 493-497, 1956

ヒヨリンエステラーゼの組織化学

——後染色及分布の特異性について——

沖中 重雄・吉川 政己・宇尾野公義・室 隆 雄
井形 和弘・田 辺 等・上 田 敏・朝長 正徳

東京大学医学部第三内科（主任：教授 沖中 重雄）

Histochemistry of Cholinesterase-Devices of Counterstaining and Specific Distribution

Shigeo OKINAKA, Masaki YOSHIKAWA, Masanori UONO,
Takao MURO, Akihiro IGATA, Hitoshi TANABE,

*From the third Department of Internal Medicine (Chief: Prof. S. Okinaka)
Tokyo University School of Medicine, Tokyo*

Introduction

The histochemical investigation of cholinesterase (ChE) concerning the nerve-activity is very important, giving a morphological basis to the neurohu-