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Further study on the activity specific for p-amino-salicylic acid and isonicotinic acid hydrazide as electron donor in enzymatic reduction of NAD*

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In the enzymatic reduction from nitrate to ammonia, nitrate, nitrite, hyponitrite or hydroxylamine serves as electron acceptor from $NADH_2$ through FAD (Yamafuji, Osajima, Omura 1960 ; Yamafuji, Osajima, Omura, Hatano 1960). Oximes and γ-glutamylhydroxamic acid (GHA), probably =NOH group in the molecule, similarly play in the reactions of oximase (Yamafuji, Osajima, Omura, Yoshida 1959) and GHA reductase (Omura, Ikeda, Osajima, Kobayashi, Kanegae, Yamafuji 1968) discovered by us. These enzymes as well as 4 reductases in the nitrate reducing system have common properties except inhibition by several substances. On the other hand, an enzymatic system reducing nitrophenol to aminophenol was demonstrated in pig heart by Otsuka and Honda (1961). Analogous reduction of 2, 4-dinitro-1-naphthol-7-sulfonic acid (a yellow pigment formerly employed in food processing) to diamino derivatives by fungal enzyme was also elucidated by Akuta (1962). These nitro reductions were demonstrated to be conducted in the same mechanism with that of bacterial nitrate reductase. Egami, Ebata and Sato (1951, 1952) found that nitro group in chloramphenicol is reduced to amino group by cell free enzyme solution of nitrite reductase prepared from *Streptomyces hemolyticus*. Another examples of nitro reduction were reported by several workers, too (Zucker, Nason 1955, Higgins 1961, Villanueva 1964). These observations suggest that there might be close resemblance between enzymatic reductions of inorganic nitrate and orgaic nitro compounds.

Contrary to the nitrate reduction, ammonia, hydroxylamine, hyponitrite or nitrite is an electron donor to NAD in the oxidation of ammonia

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to nitrate, accompanying formation of NADH, (Yamafuji, Osajima, Omura 1960, Yamafuji, Osajima, Omura, Hatano 1960). Like the nitrate reducing system, 4 deyhdrogenases have also general properties with some exceptions. Furthermore, it was suggested that urea may be dehydrogenated in the same way, donating electron to NAD, by urea dehydrogenase which had been demonstrated in the enzyme solution of the ammonia dehydrogenating system and have the similar properties (Omura, Osajima 1961 a, 1961 b, Omura, Osajima, Tsukamoto 1966). Considering these dehydrogenases, in addition to inorganic and organic nitro reductions, it is presumed that dehydrogenation of organic amino compounds might be also found when the same procedure had been applied to them. In fact, as reported in the preceding paper (Omura, Osajima, Uchio, Nakamura 1969b), enzymatic activities which catalyse NAD reduction in the presence of p-aminosalicylic acid (PAS), isonicotinic acid hydrazide (INAH), a-ketoglutarate oxime (KGO) and GHA as electron donor were confirmed in green algae. They were respectively proposed to be "PAS : NAD oxidoreductase", "INAH: NAD oxidoreductase', "KGO: NAD oxidoreductase" and "GHA: NAD oxidoreductase", although clear evidence had not been obtained to demonstrate that the activity is attributed to the individual enzyme. However, for these activities, too, common properties were observed, while it was revealed that the activities specific for PAS and INAH are different from those for KGO and GHA by examining the extraction of the activities from dry powder of green algae. Both PAS and INAH have some physiological functions such as antituberculous activity and inhibiting effect on germination or growth of certain plant (Sato 1961). On the other hand, some suggestion of amino oxidation had been also already presented (Trebst, Pistorius, Baltscheffsky, 1967). In the present experiment, therefore, it was further examined whether or not the activity for PAS is different from that for INAH, with some concomitant supplemental study.

Experimental

According to the procedure described in the foregoing paper (Omura, Osajima, Hatano, Mineura 1969), the enzyme solution was usually prepared as follows : About 5 g algal powder was suspended in 0.05 M Tris-HCl buffer and sonicated at 10 KC for **20** minutes in a Kubota's sonic oscillator in cold. After keeping the homogenate at 35°C for 60 minutes, it was centrifuged at 12,000 r. p. m. for 30 minutes to obtain the extract. From the clear extract, enzyme was precipitated with cold acetone between 33 % and 75 %. The pellet collected was dissolved in cold H_2O and the solution was dialysed against running water.

By the similar procedure, the enzyme solutions were also prepared from extract of fresh animal tissues.

Efficiency of PAS and INAH as electron donor was spectrophotometrically assayed by estimating NADH, formation which was conducted under the routine condition in the dehydrogenase system (Omura, Osajima, Asano, Seiki 1969). The activity was expressed by the increase of the optical density at 340 mμ for 30 minutes per mg protein N.

Result and Discussion

It was already confirmed that the enzymatic increase of the optical density at 340 mμ with PAS or INAH was attributed to NADH, formation which was decreased by alcohol dehydrogenase and acetaldehyde (Omura, Osajima, Uchio, Nakamura 1969a). A progress of the reaction was then determined by estimating the optical density during the incubation at 35°C for 5, 10, 15, 20, 25, 30 and 60 minutes with PAS or INAH and without adding them. On the other hand, the activity was assayed with varying volume of the enzyme solution from 0.5 ml. to 2.0 ml. Both Figs. 1 and 2 indicate that the formation of NADH, is approximately proportional with the reaction time or the quantity of the enzyme. However, difference was not observed between PAS and INAH, although the NAD reduction is a little higher with the latter than that with the former.

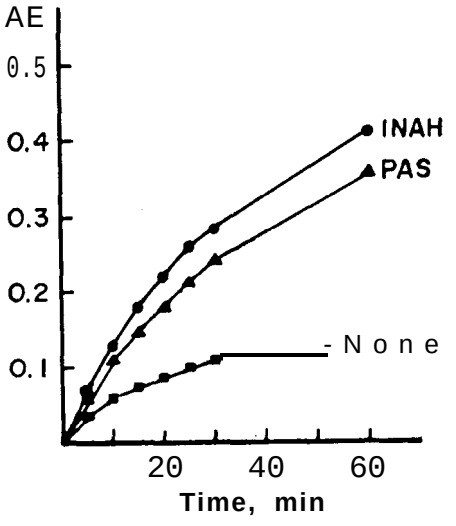

Fig. 1. Progress of the reaction.

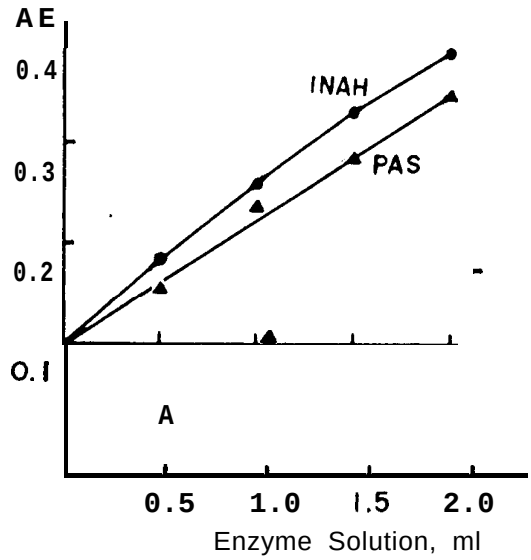

Fig. 2. Reaction with varying volume of the enzyme solution.

While the optimum pH of about 7.5 had been equally observed for both the activities with PAS and INAH, stability of them at various pH's is more or less different, as shown in Fig. 3. In this experiment,

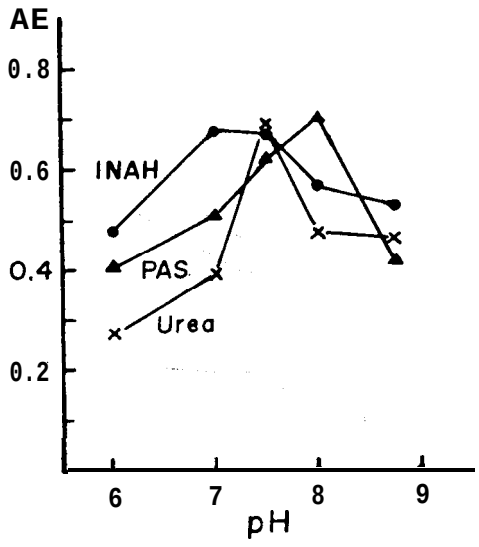

Fig. 3. Stability of the activity at various pH.

the enzyme solution was adjusted to a desired pH with 0.2 M HCl or 0.2 M NaOH, kept at 0°C for 60 minutes and the remaining activities were estimated after readjusting it to pH 7.5. Stability of urea dehydrogenase was also determined for comparison. Thus, it was shown that the activity specific for PAS is most stable at pH 8 and that for INAH at pH 7–7.5, whereas urea dehydrogenase at pH 7.5.

The influence of several inhibitors on algal enzyme was estimated as usual way and the result is indicated in Table 1 with that on urea dehydrogenase.

Table 1. Effect of several inhibitors.

Inhibitor	Conc. M	PAS	INAH	Urea
Sodium azid	5×10^{-3}	100	55	100
	5x10-4	45	15	40
KCN	5×10^{-3}	57	15	100
	5×10^{-4}	7		54
EDTA	5×10^{-3}	30	13	
	5×10^{-4}	0	10	33
Thiourea	5×10^{-3}	60	20	100
	5×10^{-4}	45	17	50

Figures indicate inhibition % and-is not examined.

Although all the activities are inhibited by sodium azide, potassium cyanide, ethylenediaminetetraacetate and thiourea, the activities for PAS and INAH are generally less retarded than urea dehydrogenase. In addition, inhibiting rate on the activity for PAS seems to be higher than that for INAH, while distinctive difference could not be established.

Concerning the enzymatic cycle between ammonia and nitrate in liver (Yamafuji, Osajima, Omura, Hatano 1960), inorganic phosphate plays a peculiar and essential role in determining the activity, because phosphate inhibits 4 dehydrogenases from ammonia to nitrate, while it is necessary for 4 reductases in the reducing system from nitrate to ammonia. Therefore, the activities were estimated with PAS and INAH in the presence of phosphate and shown in Table 2. In this experiment, 0.5 ml phosphate buffer (pH 7.5) of various concentration was added to the reaction mixture of 1 ml enzyme solution, 1 ml 0.025 % NAD solution, 0.5 ml 0.2 M Tris buffer (pH 7.5), 0.1 ml 0.1 M PAS or INAH and 0.9 ml H_2O and the change of the optical density was estimated.

Table 2. Effect of phosphate.

PO_4, M	0	10^{-4}	10^{-3}	10^{-2}	2.5x10-2
PAS	0.720	0.720	0.784	0.800	0.900
INAH	0.472	0.440	0.388	0.352	0.360

Table 2 indicates that the activity for PAS is more or less enhanced by phosphate, whereas that for INAH is retarded, differing from urea dehydrogenase which is activated by phosphate in a small amount but inhibited by it of higher quantity.

In the foregoing paper (Yamafuji, Osajima 1963), it was confirmed that 4 dehydrogenases are not adsorbed by DEAE cellulose, while 4 reductases from nitrate to ammonia, oximase and GHA reductase are adsorbed and eluted by sodium acetate between 10^{-3} M and 10^{-1} M. Thus, separation of the dehydrogenases from the reductases could be effectively accomplished and all the enzymes were incidentally purified. Urea dehydrogenase of green algae was similarly not adsorbed on DEAE cellulose (Omura, Osajima, Uchio, Nakamura 1969 a). Therefore, the enzyme solution of acetone precipitate from algal extract was put on a DEAE cellulose column equilibrated with 10^{-3} M sodium acetate, washed with the same solvent, eluted with it of higher concentration of 10^{-1} M and dialyzed for 3 hours against running water. The activity was comparatively assayed with that of original algal extract and the enzyme solution prepared from the acetone precipitate.

Table 3. Treatment of the enzyme solution with DEAE cellulose.

Fraction	Original Extract	Acetone Precipitate	Not Adsorbed	Extract with Na acetate
N, μg	533	150	76	19
PAS	0.281	0.620	1.846	0
INAH	0.446	0.482	2.171	0

As evident in Table 3, it was shown that both the activities for PAS and INAH are not adsorbed with DEAE cellulose, similar to 4 dehydrogenases for ammonia, hydroxylamine, hyponitrite and nitrite as well as urea dehydrogenase.

Although similarity had been observed with respect to many properties of the activities specific for PAS and INAH of green algae, pH stability and the effect of inorganic phosphate are evidently different each other. These differences give the unequivocal support for the assumption that the enzymatic activity is attributed to the peculiar independent enzyme. Therefore, as presumed before, the enzyme must

be "PAS : NAD oxidoreductase" and "INAH: NAD oxidoreductase" respectively. Because NADH, is enzymatically formed from NAD in the presence of PAS and INAH, these compounds might be probably dehydrogenated Thus, in other words, the enzymes might be PAS dehydrogenase and INAH dehydrogenase, although the products had not been identified.

In addition to fowl liver mainly employed, these activities were investigated with several animal tissues such as liver of cattle, and liver, kidney and pancreas of rabbit. The enzymatic activities were estimated not only with PAS and INAH but also with urea and GHA which had never been estimated for these animal tissues.

Table 4. Activities in some animal tissues.

Tissue	PAS	INAH	Urea	GHA
Fowl liver	0.070	0.057	0.034	-
Cattle liver	0.388	0.323	0.273	-
Rabbit liver	0.134	0.160	0.090	0.230
Rabbit kidney	0.070	0.101	0.045	0.036
Rabbit pancreas	0.007	0.010	-	0.287

-: The activity was not examined.

Table 4 shows that the activities are contained in these tissues, while only the activity for GHA was detected in rabbit pancreas in which those for PAS and INAH had seldom been demonstrated.

Summary

Some supplemental experiments were carried out on the activities specific for PAS and INAH as electron donor in the enzymatic reduction of NAD. Similarity was observed in inhibition by sodium azide, potassium cyanide, ethylenediaminetetraacetate and thiourea, although inhibition rate is more or less different. In addition, the enzymatic activity was not adsorbed on DEAE cellulose as urea and 4 dehydrogenases in the ammonia dehydrogenating system. However, clear evidence to show the difference was obtained in pH stability and influence of inorganic phosphate on the activity. The activity specific for PAS is most stable at pH 8 at 0°C for 60 minutes and enhanced by phosphate. On the other hand, maximum stability of the activity for INAH was observed at pH 7-7.5 and retarded by phosphate. These properties are different each other and from that of urea dehydrogenase which is most stable at pH 7.5 and activated by phosphate in a small amount but inhibited by it of higher quantity. Thus, the enzymes

were proposed to be "PAS : NAD oxidoreductase" and "INAH: NAD oxidoreductase" or alternatively presumed to be dehydrogenases of PAS and INAH.

These activities as well as dehydrogcnases of urea and GHA were demonstrated in several animal tissues such as cattle liver and liver, kidney and pancreas of rabbit, too.

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