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CONJUGATED NORMETANEPHRINE IN HUMAN AND RAT PLASMA AND ERYTHROCYTES

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Abstract—Enzymatic hydrolysis was applied to the deconjugation of normetanephrine (NMN) in plasma and red blood cell lysate. By this procedure, in human plasma 77% of total NMN circulated in sulfate-conjugated form, while in rat plasma 63% was in glucuronidated form. Total NMN in human lysate was significantly higher than in plasma ($P < 0.001$) and was mostly in the free form, indicating that red blood cells may play an important role in metabolism of norepinephrine. Enzymatic hydrolysis is superior to the standard method by acid hydrolysis plus heat since: (1) more conjugated NMN is hydrolyzed in human plasma and (2) a smaller sample is needed for hydrolysis.

The level of norepinephrine (NE) in plasma is used as an index of sympathetic nerve activity. However, free plasma NE represents only a small fraction of released NE. A great percentage of released NE is taken into the neuron or metabolized. Catechol-*O*-methyltransferase (COMT) is the extraneuronal enzyme that methylates NE, thereby converting it to normetanephrine (NMN). Therefore, measurement of NMN supplies us with additional information about sympathetic nerve activity.

NMN is excreted in conjugation with glucuronic acid in rats [1] and with sulfate in humans [2, 3]. Twenty years ago, Smith and Weil-Malherbe [3] reported that sulfatase hydrolysis gave higher results than acid hydrolysis (pH 1, 100°, 20 min) for metanephrine and NMN in human urine. Nevertheless, acid hydrolysis has been used for estimation of total NMN [2, 4–7] because sulfatase hydrolysis was considered time-consuming. Moreover, in most of the published methods [2–7] hydrolysis of conjugates is usually carried out before NMN is assayed. Our recent success with the simultaneous enzymatic hydrolysis of conjugated catecholamines (CA) and radio enzymatic assay of free CA [8] encouraged us to apply this approach for deconjugation of NMN sulfates and glucuronides.

Recently, we documented that red blood cells (RBC) contain higher levels of total NMN than plasma and suggested that RBC might be an important side for catabolism of NE [8]. In order to clarify further the disposition of NMN in RBC, our method for deconjugation was applied to RBC lysate.

MATERIALS AND METHODS

Materials. β-Glucuronidase (from *Escherichia coli*, Type IX, 840,000 units/g solid) and sulfatase (from Aerobacter aerogenes, Type VI, 3.2 units/mg protein) were purchased from the Sigma Chemical Co. (St. Louis, MO). The preparation of phenylethanolamine-*N*-methyltransferase (PNMT) is described elsewhere [6]. Organic solvents for two-dimensional thin-layer chromatography were obtained from the Fisher Scientific Co. (Fair Lawn, NJ). All other materials have been mentioned previously [6, 9]. Male Wistar rats, weighing 280–300 g, were purchased from Simonsen Laboratories (Gilroy, CA).

Sample collection. Rats were housed in separate cages and received food and water *ad lib*. They were anesthetized with ether, and a polythene catheter filled with heparinized saline was inserted through the femoral artery with the tip above the aortic bifurcation and the other end exteriorized at the back. Twenty-four hours later, an extension was connected to the end of the catheter, and a 2-ml blood sample was collected while rats were at rest in home cages. Human venous blood samples were obtained in our laboratory from healthy, ambulatory volunteers aged 30–55 years.

Blood samples were mixed immediately in ice cold tubes with a solution of glutathione (GSH) and ethylenebis tetraacetic acid (EGTA), pH 5.5, 20 μl/ml. One milliliter of each sample was centrifuged, and the plasma was stored at −70° until time of assay.

Another 1 ml of blood was used for RBC lysate preparation as described previously [9]. In short, when 1 ml of blood was layered on an equal volume of Ficoll and centrifuged, RBC collected at the bottom. RBC were washed three times with 4 ml of physiological saline. Packed, weighed RBC were hemolyzed with 4 vol. of deionized water containing GSH and EGTA. The lysate was centrifuged once more, and the membrane-free portion was stored at −70° until assay.

Method. Free NMN plasma and lysate was determined by radioenzymatic assay based on the conversion of NMN to its N-methylated derivative, metanephrine, using PNMT from bovine adrenal medulla and [^{3}H]*S*-adenosyl-1-methionine ([^{3}H]SAM) [6].

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To measure total NMN by acid hydrolysis, 250 μl of plasma plus 20 μl of 5 N perchloric acid was mixed in a glass-stoppered conical tube and heated in boiling water (98°) for 20 min. After centrifugation, the pH of the supernatant fraction was adjusted by adding 20% of 5 N Tris base. Then total (free and conjugated) NMN was determined by the same method as free NMN.

Our preliminary experiments showed that human plasma had predominantly sulfated NMN while that of rat had mainly glucuronidated NMN. In human plasma, glucuronidated NMN did not exist at significant levels (free: 837 ± 63 pg/ml, free plus glucuronidated NMN: 862 ± 86 pg/ml, N = 6 humans), nor did sulfated NMN in rat plasma (free: 708 ± 27 pg/ml, free plus sulfated NMN: 746 ± 45 pg/ml, N = 6 rats). Consequently, sulfatase and β-glucuronidase were used for hydrolysis of conjugated NMN in humans and rats, respectively. In human samples, hydrolysis was carried out in a reaction mixture where N-methylation of NMN was catalyzed by PNMT in the presence of [^{3}H]SAM during incubation for 60 min at 37°, pH 8.4. On the other hand, rat samples were preincubated with β-glucuronidase for 30 min, and then total NMN was measured.

Usually three blanks were used for the assay of free and conjugated normetanephrine. One blank was run without sulfatase and β-glucuronidase for free normetanephrine. The other two blanks were run with sulfatase and β-glucuronidase for sulfated and glucuronidated normetanephrine respectively. Neither of the latter blanks increased blank counts, suggesting that these enzymes did not contain normetanephrine.

Two-dimensional thin-layer chromatography was performed to eliminate the possibility of contamination by analogous substances that might contribute to higher radioactivity in enzymatic deconjugation. The plate was developed as follows: tertiary amyl alcohol–methylamine–toluene (60:30:20) in the first direction for 100 min and chloroform–methanol–1 N ammonium hydroxide (60:35:5) in the second direction for 80 min [6, 10].

The results are expressed as mean ± S.E.M. and statistically analyzed by means of a paired and unpaired Student's *t*-test.

RESULTS

Since sulfatase has relatively high activity even at pH 8.4 [11], enzymatic hydrolysis of human samples was carried out in the reaction mixture for N-methylation. Preliminary studies showed that 20 milliunits of sulfatase was able to maximally hydrolyze conjugated NMN in 25 μl of sample within 60 min without affecting internal standard and blank counts.

On the other hand, in rat samples where β-glucuronidase was added directly to the reaction mixture, only a small increase of NMN was found compared to samples without the enzyme. This indicated that β-glucuronidase did not completely hydrolyze conjugated NMN at pH 8.4, the optimum pH of PNMT. Thereafter all rat samples were preincubated with β-glucuronidase at pH 7.4 [12]. Then N-methylation was done at pH 8.4 by adding PNMT, [^{3}H]SAM and 0.4 M Tris. Figure 1 shows that 100 units β-glucuronidase was suitable for maximal hydrolysis of rat conjugated NMN since plasma NMN reached a plateau (lower curve) and the internal standard was minimally inhibited (upper curve). As shown in Fig. 2, a 30-min preincubation gave maximum hydrolysis of rat glucuronidated NMN.

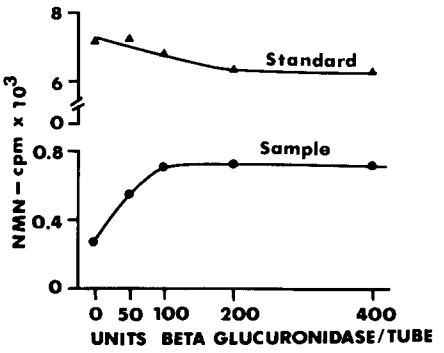

Fig. 1. Hydrolysis of NMN glucuronides in rat plasma and inhibition of N-methylation of NMN standard (500 pg free base) by increasing the amount of β-glucuronidase. NMN was N-methylated to [^{3}H]metanephrine in the presence of PNMT and [^{3}H]SAM (ordinate).

Table 1 shows that 77% of human plasma NMN was conjugated as determined by enzymatic hydrolysis. In lysate, the mean for total NMN was higher than that for free NMN, but the difference was not statistically significant. Therefore, it seems that NMN in RBC exists mainly in free form and at significantly higher levels than in plasma. When the results of enzymatic hydrolysis were compared to those of acid hydrolysis, the former gave significantly higher values than the latter ($P < 0.001$). Two-dimensional thin-layer chromatography (Fig. 3) showed only one distinct peak of radioactivity at the position of NMN, indicating absence of contamination.

In rat plasma, 63% of NMN existed in conjugated form (Table 2). Though the results by enzymatic hydrolysis were higher than those by acid hydrolysis plus heating, the difference was not statistically significant (Table 2).

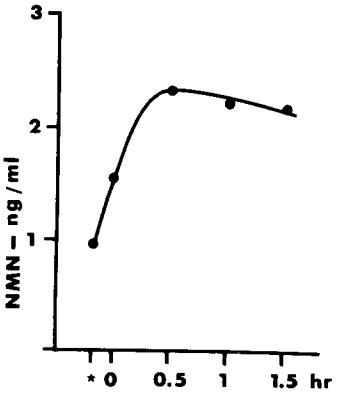

Fig. 2. Time course of hydrolysis of NMN conjugates in rat plasma by β-glucuronidase (100 units/tube). The asterisk (*) indicates that β-glucuronidase was not added to this sample.

Table 1. Human plasma and RBC lysate NMN*

		Plasma	Lysate
Free	pg/ml	870 ± 81	7410 ± 1038†
Total (enz. hyd.)	pg/ml	3709 ± 213‡	8392 ± 1055 (NS§)
Conjugated	pg/ml	2839 ± 161	982 ± 640
Conjugated	%	76.7 ± 1.3	11.2 ± 8.0 (NS)
Total (acid hyd.)	pg/ml	2266 ± 116‖	¶
Conjugated	pg/ml	1386 ± 109	¶
Conjugated	%	61.7 ± 3.0	¶

* Values are expressed as mean ± S.E.M., N = 6.
† P versus plasma: $P < 0.001$.
‡ P versus free: $P < 0.001$.
§ P versus free: not significant.
‖ P versus enzymatic hydrolysis: $P < 0.001$.
¶ Cannot be measured by acid hydrolysis.

DISCUSSION

A method for enzymatic hydrolysis of conjugated NMN and measurement of total NMN by radioenzymatic assay was described here. Since sulfatase has relatively high activity at pH 8.4 [11], we could accomplish hydrolysis of sulfate-conjugate NMN in human plasma and RBC lysate by adding sulfatase directly into the reaction mixture of the radioenzymatic assay for NMN. For rat plasma, addition of β-glucuronidase to the radioenzymatic reaction mixture resulted in significantly less total NMN than with acid hydrolysis (1208 ± 61 versus 2331 ± 166 pg/ml, N = 5) because β-glucuronidase has optimal activity at lower pH than used for radioenzymatic assay [13]. Preincubation of rat sample with β-glucuronidase at pH 7.4 for 30 min resolved the problem (Figs. 1 and 2) and gave results similar to those of acid hydrolysis. CA glucuronides are usually considered to be stable in boiling acid [8, 14]. However, our results showed that NMN glucuronides were different from CA glucuronides in this respect and easily hydrolyzed by boiling acid as well as by β-glucuronidase. This may suggest that, in acid, conjugation at the para position is not as stable as that at the meta position. Although enzymatic and acid hydrolyses of rat samples take about the same time to complete, the amount of sample needed for the former is only 25 μl compared to 250 μl for the latter. Therefore, when sample volume is small, enzymatic hydrolysis is preferable to acid hydrolysis.

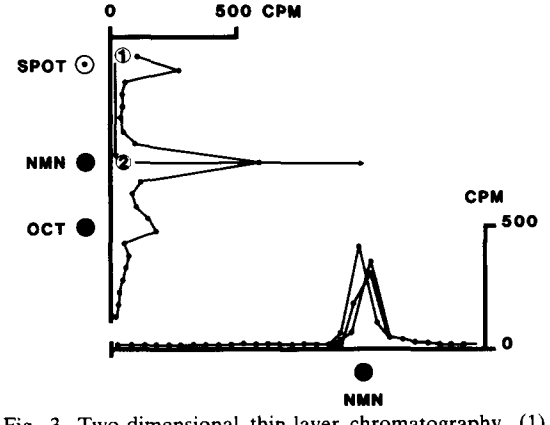

Fig. 3. Two-dimensional thin-layer chromatography. (1) The distribution of tritiated products of rat plasm simple after deconjugation and N-methylation. SPOT: original spot for thin-layer chromatography. NMN and OCT: normetanephrine and octopamine which were N-methylated to [^{3}H]metanephrine and [^{3}H]synephrine respectively. (2) After second development of NMN ([^{3}H]metanephrine) spot, only one peak of radioactivity was detected.

In human plasma, significantly more total NMN was obtained by enzymatic than by acid hydrolysis (Table 1). Possible reasons are: (1) inhibition of PNMT by perchloric acid, and/or (2) incomplete hydrolysis in acid. With regard to the first, in our assay, recovery of an NMN standard added to a sulfatase-treated sample had the same counts as that of a standard in water and significantly more than one in an acid-hydrolyzed sample. These results suggest that PNMT could be inhibited by perchloric acid as has been reported for COMT [15]. Incompleteness of acid hydrolysis was indicated by finding that the addition of sulfatase to an acid-hydrolyzed sample increased the sample count (unpublished observation). Smith and Weil-Malherbe [3] also reported that the combination of acid hydrolysis and sulfatase incubation yielded results similar to those obtained with the sulfatase alone but consistently and significantly higher than results of acid hydrolysis alone. Therefore, hydrolysis in acid may be incomplete. Moreover, the greater amount of total NMN assayed after enzymatic hydrolysis was not due to contamination by analogous substances, e.g. 3-methoxytyramine, as indicated by two-dimensional thin-layer

Table 2. Rat plasma NMN*

		Plasma
Free	pg/ml	746 ± 71
Total (enz. hyd.)	pg/ml	1966 ± 149
Conjugated	pg/ml	1220 ± 93 (NS†)
Conjugated	%	62.5 ± 2.1
Total (acid hyd.)	pg/ml	1868 ± 295
Conjugated	pg/ml	1122 ± 254
Conjugated	%	56.4 ± 3.9

*Values are expressed as mean ± S.E.M., N = 8.
† P versus acid hydrolysis: not significant.

chromatography (Fig. 3). The results shown in Fig. 3 also indicate that free NMN was not contaminated. Thus, the high free NMN values, compared to those reported previously by others [7], probably result from improved techniques as well as quality of available isotopes [^{3}H]SAM).

Since the main conjugate of NE is sulfate in rat plasma [8], we expected that NMN also would be mainly sulfate conjugated, because NMN has stronger affinity for phenosulfotransferase (PST) than NE due to the meta substituent on the phenolic ring [16]. However, the main conjugate of rat NMN was glucuronide. Taken in conjunction with the locations of PST and UDP-glucuronyl transferase and the affinities of amines to them, our observations may provide new clues as to the main sites of sulfation and glucuronidation for catecholamines and their metabolites.

As we reported previously [9], NMN in RBC was present in significantly higher concentration than in plasma. In this study we have demonstrated the existence of RBC NMN mostly in free form (Table 1). This result supports our speculation that RBC may be an important site for CA catabolism [9], i.e. NE transported into RBC may be converted into free NMN.

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