MICROBIOLOGICAL ASSAY OF PANTOTHENIC ACID IN BLOOD AND URINE

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The quantitative determination of pantothenic acid (PaA) so far devised can be classified into two methods, *i. e.*, chemical and microbiological. The former (1) is not sensitive enough for the measurement of PaA values of less than 1 mg/ml. On the other hand, the latter is suitable for both urinary and blood PaA, since it is possible to estimate the amounts of the vitamin as low as 10 μ g/ml. The microorganisms available for the assay of the vitamin are *Saccharomyces carlsbergensis* (2), *Proteus morganii* (3), *Streptococcus faecalis* (4, 5), and *Lactobacillus arabinosus*. Among them, *Lactobacillus arabinosus* seems to give the least drift and good recovery for the assay (6—8). For this reason, this organism was used in the present investigation. However, it should be kept in mind in the use of this organism for micrological assay that the organism fails to utilize the bound PaA such as pantethine, phosphopantethine and coenzyme A. Therefore, the bound forms have to be degraded into the free form preliminarily by enzymatic treatment for the determination of PaA.

A number of researches (7) have hitherto been made on the PaA values in urine, whereas only a little has been done on the values in blood (8). Regarding the quantitative determination of the vitamin in blood so far presented by other investigators, there are roughly two investigations which contributed much to the assay, *i. e.*, by Pennington (9) and by Fredrickson (10). The former deals with the blood PaA, referring only to the free form, and the latter with the free and bound forms. Recently, it was found that the majority of the total PaA existed in the blood as the bound, active form.

It suggests that for the exploration of PaA metabolism, it is necessary to determine not only the free, but also the bound form. Thus the free and bound forms of the vitamin were quantitatively measured in the blood by the present author.

EXPERIMENTAL

The method used in this study is partially the same as described in the Pharmacopeia of the United States (12).

Materials

1. Standard Stock Solution of Calcium Pantothenate (12)

2. Medium

Stock Culture Medium—The medium (Table I) pertinent to the PaA assay was employed in this study. A 6% solution of the medium was made and autoclaved at 15 pounds (120°). A fresh stab of the stock culture was prepared every week and was used for inoculation.

TABLE I
Stock Culture Medium

Component	Content	Component	Content
Yeast extract	5 g	Sodium acetate	10 g
Peptone	10 g	MgSO ₄ ·7H ₂ O	0.1 g
Glucose	10 g	MnSO ₄ ·7H ₂ O	5 mg
KH ₂ PO ₄	0.25 g	FeSO ₄ ·7H ₂ O	5 mg
K ₂ HPO ₄	0.25 g	Agar	20 g

*Basal Medium*¹—As shown in Table II, 7.7 g of powdered basal medium was dissolved in 100 ml of distilled water. 2.5 ml-aliquots of the solution and 2.5 ml of the standard solution of the sample were pipetted into the test tubes and was autoclaved at 15 pounds.

TABLE II
Basal Medium

Component	Content	Component	Content
Acid-hydrolyzed casein	10 g	<i>p</i> -Aminobenzoic acid	200 µg
L-Cysteine	400 mg	Nicotinic acid	400 µg
DL-Tryptophane	200 mg	Ryridoxine hydrochloride	800 µg
Adenine sulfate	20 mg	KH ₂ PO ₄	1 g
Guanine hydrochloride	20 mg	K ₂ HPO ₄	1 g
Uracil	20 mg	MgSO ₄ ·7H ₂ O	400 mg
Thiamine hydrochloride	200 µg	MgSO ₄ ·4H ₂ O	20 mg
Riboflavin	400 µg	Sodium acetate	20 g
Biotin	0.8 µg	Glucose	40 g

Inoculation Medium—Inoculation medium used in this study is the stock medium, from which agar has been excluded.

Sterilization of a 4% solution of the inoculation medium was likewise made similarly to the basal and stock culture media.

3. Inoculum

On the day before inoculation, the organism was transferred to the inoculation medium from a stock culture medium of *Lactobacillus arabinosus*, and was incubated for 24 hours at 37°.

Thereafter, the organism grown on the inoculation medium was washed repeatedly with sterilized physiological saline solution by centrifugation, and then adjusted the suspension to the transmittance of 60–90%.

The organism suspension thus obtained is the inoculum.

¹ Made with the preparation of Nissan Suisan Company or that of Difco Laboratories.

Methods

One drop of the inoculum was added to the test tube containing the sterilized solutions of 2.5 ml basal medium and 2.5 ml standard solution (40 μg of PaA per ml) or sample. Incubation was made for 72 hours at a temperature of 37°.

For the purpose of measuring the lactic acid, produced during the bacterial growth in the tube, acidometry was carried out with 0.1 *N* NaOH using phenolphthalein as an indicator. Fig. 1 is a standard curve obtained by using the standard media.

FIG. 1 Standard Response Curve Determined after 72 Hours

1. Determination of Blood Pantothenic Acid

Treatment of Blood for the Measurement of the Free Form—Blood was withdrawn from the antecubital vein, and oxalate mixture was immediately added to the blood in proportions of 5–10 mg per 5 ml of blood, and the mixture was kept in the frozen state. For the measurement of the free form of the vitamin, it was diluted with an adequate amount of water at room temperature.

Each of 0.5, 1.0 and 2.0 aliquots of the solution was pipetted into the test tubes and water was added to a final volume of 5.0 ml.

Treatment of Blood for the Measurement of Total PaA—One ml of oxalated blood was added to 0.4 ml of the solution diluted to half the concentration of Lipmann's original enzymatic solution (13–16). This mixture was incubated for 24 hours at 37°, and was diluted with water adequately.

Preparation of Lipmann's original enzymatic solution (13–16): Extract of pigeon liver, 2% intestinal phosphatase solution and 1 *M* Tris hydroxymethyl aminomethane (pH 8.3) mixed in a proportion of 2:1:1.

Bound PaA was calculated as the difference between the total and the free values.

2. Technical Consideration for Blood PaA Determination

Relation of the Added Amount of Oxalate Mixture to the Recovery—The effects of oxalate mixtures at various concentrations on the recovery of the known amounts of PaA was scarcely recognizable so long as the amounts of oxalates added to the medium were below 7 mg (Table III).

TABLE III
Effect of Oxalate Mixture on Pantothenic Acid Values
Oxalate mixture consisting of 62.5% ammonium oxalate and 37.5% potassium oxalate was used.

Oxalate mixture	Pantothenic acid value
mg/ml	mg/ml
0	40
1	40
2	40
3	40
4	40
5	40
6	42
7	43

This result supports the validity of the author's method, *i. e.*, the effect of the added oxalate mixture was quite negligible, since the blood sample was so diluted that it contained less than 2 mg of the mixture.

Influence of the Freezing of Blood on PaA Recovery—The PaA estimations were carried out on the samples as cold as possible in order to avoid its autolysis. The samples were kept below 0° in the refrigerator to freeze for various times in order to test any effect of freezing time on the recovery of the PaA value. The determinations were performed at each time interval of freezing. It was found that no fluctuations of the values occurred if the freezing time was within 30 days. In this study the blood PaA values were determined within 30 days after venesection.

Influence of Autolysis—Blood samples were kept at room temperature or at 37° for various hours (Table IV). After 72 hours, the PaA value recovered from the sample increased up to 273 mμg/ml, indicating a marked increase of the free form as compared with the value determined immediately after venesection. This increase may probably result from autolysis. Therefore, the free form was measured as soon as possible to avoid the effect of autolysis.

TABLE IV
Effect of Autolysis on the Pantothenic Acid Levels in Blood

Condition		Pantothenic acid levels in blood after hours						
Temperature	pH	0	3	12	24	48	72	96
°C		mμg per ml						
16, 27	7.0	25	29	42	101	143	161	164
37	7.0	25	48	71	199	254	273	257

TABLE V
Time Necessary to Exert Enzymatic Activity

Sample No.	Pantothenic acid levels in blood after hours				
	0	4	8	16	24
			<i>mg per ml</i>		
1	23	398	429	434	435
2	25	411	437	435	441
3	27	418	484	490	483
Mean	25	409	450	453	453

Duration of Time Necessary to Exert Enzymatic Activity—Blood sample were treated according to Lipmann for various periods. As shown in Table V, it was suggested that the enzymatic treatment should be made at least for 16 hours. Thus the enzymatic treatment for 24 hours was adopted by the author throughout the study.

Effect of Concentration of Enzyme—As shown in Table VI, it was found that the treatment with the enzymes of half the concentration of Lipmann's original solution gave the least fluctuation for PaA values and besides the enzymatic activity. The method was proved to be satisfactory for the determination. Accordingly, half the concentration of Lipmann's original solution was used for the measurement.

TABLE VI
Effect of Concentration of Enzyme

Sample No.	Lipmann's enzymatic solution		
	Standard concentration	Half concentration	1/4 concentration
	<i>Pantothenic acid levels in blood (mg per ml)</i>		
1	405	430	425
2	450	440	420
3	435	450	445
4	440	425	410
Mean	432.5	432.5	425.6

3. Determination of Free and Bound Forms of PaA in Urine

Treatment of Urine—Twenty four hour-urine was kept by adding toluene until used. The collected urine was not subjected to freezing or mixed with oxalate mixture. The determination of the free form in the urine was made without enzymatic treatment.

4. Technical Consideration of Urinary PaA Determination

Influence of Light and Temperature on Urinary PaA—The collected urine was divided into two parts. One was kept 30 cm below a 5-watt electric bulb at 37°, while the other was kept in a dark room at 0° for 24 hours. The PaA values obtained from the two urine samples were approximately identical, 5.4 mg/ml. This fact shows that the urinary values are influenced neither by light nor by temperature. So the urinary PaA determination was made at room temperature in the ordinary laboratory.

RESULTS

1. PaA Levels in the Total Blood of Healthy Subjects

A survey was made on 30 healthy persons of both sexes whose ages ranged from 15 to 50. Table VII shows the PaA values of the whole blood in the fasting state. The total value was found to be 364.5 to 540.0, with an average of 464.2 m μ g/ml. The bound form was 351.0 to 490.0, with a mean of 435.5 m μ g/ml, while the free form was 28.7 on the average, ranging from 14.0 to 70.0 m μ g/ml.

TABLE VII
Pantothenic Acid Levels in Normal Persons

No.	Age	Sex	Pantothenic acid levels						
			Free	Bound	Total	B/T	Free	Bound	Total
			Blood				Urine		
			m μ g per ml				m μ g per day		
1	20	m	40	420	460	0.91	3.53	0.00	3.53
2	30	m	40	410	450	0.91	3.44	0.01	3.45
3	31	m	60	450	510	0.88	3.06	0.03	3.09
4	32	m	40	410	450	0.91	2.67	-0.01	2.66
5	29	m	15	450	465	0.97	3.73	0.08	3.81
6	28	m	30	405	435	0.93	2.47	-0.10	2.37
7	27	m	30	480	510	0.94	2.52	-0.01	2.51
8	26	m	15	440	455	0.97	4.28	0.00	4.28
9	28	m	45	370	415	0.89	3.98	0.00	3.98
10	15	m	30	450	480	0.94	3.42	0.00	3.42
11	35	m	15	470	485	0.97	4.46	0.00	4.46
12	50	m	20	400	420	0.95	3.45	-0.01	3.44
13	42	m	25	490	515	0.95	2.54	0.02	2.56
14	41	m	70	440	510	0.86	3.35	0.02	3.37
15	17	m	60	480	540	0.89	3.25	0.03	3.26
16	25	f	15	480	495	0.97	2.57	0.01	2.58
17	23	f	20	480	500	0.96	2.46	0.01	2.47
18	24	f	15	470	485	0.97	2.54	0.02	2.56
19	25	f	25	440	465	0.94	2.52	0.03	2.55
20	27	f	40	480	520	0.92	2.58	-0.01	2.59
21	21	f	17	458	475	0.96	2.83	0.01	2.84
22	18	f	21.5	410	431.5	0.95	3.27	0.01	3.28
23	45	f	24.5	440	464.5	0.95	4.05	-0.02	4.03
24	50	f	13.5	351	364.5	0.96	3.65	0.00	3.65
25	18	f	17.5	388	405.5	0.96	3.03	0.00	3.03
26	19	f	27.5	381	408.5	0.93	3.07	0.04	3.07
27	18	f	14	381	395	0.99	3.71	-0.01	3.70
28	15	f	16.5	472	488.5	0.97	2.99	0.02	3.02
29	32	f	24	425	449	0.95	4.06	0.05	4.11
30	35	f	35	445	480	0.93	3.34	0.02	3.36
Range			14.0	351.0	364.5	0.86	2.46	-0.10	2.37
			to	to	to	to	to	to	to
			70.0	490.0	540.0	0.97	4.46	0.08	4.46
Mean			28.7	435.5	464.2	0.94	3.23	0.08	3.23
$\pm$ S. D.			± 15	± 87	± 41	± 0.2	± 0.6	± 0.03	± 0.6

2. Urinary PaA Levels in Healthy Subjects

In 30 healthy persons, the PaA levels in 24-hour urines are shown in Table VII. The total value was 2.37 to 4.46, with an average of 3.23 mg. The majority of urinary PaA was in the free form.

DISCUSSION

Quantitative measurement of PaA was carried out by a microbiological assay using *Lactobacillus arabinosus* on human blood and urine specimens. In this study, Lipmann's original enzymatic solution was diluted to half the concentration. As a result, thus diluted enzymatic solution brought forth a lower blank value, giving the least variability in PaA values. The dilution seems to have reduced the amount of PaA present in Lipmann's original solution, preserving the enzymatic activity. As for the collection of urine, almost no influence was observed on the enzymatic activity of the solution even when the urine was kept at a distance of 30 cm below a 5-watt globe at 37°. Thus it was suggested that the measurement could be successfully carried out, as long as the urine specimens were collected at room temperature.

Regarding the determination of bound PaA in the blood, Fredrickson (10) also used Lipmann's solution and found that the bound form in healthy persons was 461 ± 27 m μ g/ml on the average, a value approximately in agreement with that of the present author.

However, when a blood sample was subjected to autolysis instead of enzymatic treatment, the value obtained was much less, 273 m μ g/ml. These discrepancies were found also after different treatments of blood PaA. At any event, the enzymatic treatment of a blood sample seems to give more precise values. Therefore, the author feels it better to perform the enzymatic treatment rather than autolysis to measure more exactly the bound form of the vitamin in the blood.

As regards the free form of the vitamin in the blood, Fredrickson reported the value of 116 ± 23 m μ g/ml in healthy adults, while the present author found 28.7 m μ g/ml. But, the presents author's value obtained from the blood sample left for 48 hours at room temperature was 101 to 143 m μ g/ml, which is rather close to Fredrickson's and the increase is possibly due to autolysis. The possible explanation for this difference or accord is that, (a) presumably Fredrickson himself also has left the blood sample for more than 48 hours or so prior to the measurement, whereby the above described similarity was obtained, (b) the technique used by Fredrickson may be different from the author's, which may give rise to the difference, and (c) the difference may actually be due to the species difference.

As a matter of fact, to freeze the blood as soon as possible after the withdrawal could prevent the bound form from autolysis.

As regards the total PaA in blood, Baker (17) found the value of 470—560 m μ g/ml, whereby Clarase was used instead of Lipmann's enzymes. But this value is approximately in good keeping with the total values of 465 m μ g/ml by the author.

It has been reported by many investigators that the PaA found in the urine of healthy persons is entirely the free form. The present author's results suggest that the PaA value of the free form and the total in the urine of healthy persons is the same, since there is no bound form. There has been no report on the total PaA value obtained after enzymatic treatment of urine, though a large number of reports have been made on the free form obtained without enzymatic treatment,

as Baker (17), Schmidt (18), Wright (19), Krahnke (20), Sarett (21), Gershbery (22), Gounell (23), Pelezar (24), Burlet (25), Fitzpatrick (26), Kawai (27), and Ueshima (28). The values reported by these investigators range from 2.6 to 5.7 mg/day. The author's value of the free form was also within this range.

SUMMARY

Quantitative determination of pantothenic acid in blood and urine was made, fractionating into the free and bound forms and the following results were obtained.

1. It was found that total pantothenic acid was measurable with the use of Lipmann's enzymes, while the free form can be determined without enzymatic treatment. The bound form, therefore, could be calculated by subtracting the free form from the total value.

2. It was found that better results were obtained with half the concentration of Lipmann's solution.

3. Enzymatic treatment gave higher total values than autolysis, indicating the importance of enzymatic treatment.

4. Since the bound pantothenic acid in the blood could be degraded, in part, into the free form by autolysis, it is necessary that the measurement of the free form should be made as soon as possible to avoid autolysis.

5. The total pantothenic acid levels in the blood of 30 healthy adults were found to be 464.2 ± 41 , the bound form 435.5 ± 37 , and the free form 28.7 ± 15 $\mu\text{g}/\text{ml}$.

6. The total urinary pantothenic acid of adults was 3.24 ± 0.6 , and the free form 3.23 ± 0.6 mg/day, suggesting that the enzymatic treatment is not necessary, since urinary pantothenic acid is predominantly in the free form.

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